



Journal of  
Pathology and Bacteriology





# The Journal of Pathology and Bacteriology

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# The Journal of Pathology and Bacteriology

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## WORLD EFFECTS OF VASOPRESSIN ON THE ORGANS AND VESSELS OF RATS.

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(Plates I-IV)

It is generally accepted that the condition of eclampsia and the pre-eclamptic toxæmia are due to excessive secretions of the posterior pituitary hormone. The latter hypothesis offers an attractive explanation of the clinical and anatomical characters of these conditions, but, even after nearly twenty years, an unproven hypothesis. The claim of Anselmino and Hoffmann (1931) to have demonstrated excess of vasopressin in eclamptic blood appeared to have established the hypothesis beyond doubt, but more recent workers (Theobald, 1933-34, Byrom and Wilson, 1934, de Wesselow and Griffiths, 1934, Hurwitz and Bullock, 1935, Levitt, 1936) have failed to confirm the claim. It must be conceded, however, that such negative results do not exclude over-secretion of vasopressin, for the methods available for detecting the latter are biological and involve considerable dilution of the suspect fluid by the tissue fluids of the test animal. Cushing's (1933) contention that basophil-cell invasion of the pars posterior of the pituitary is characteristic of eclampsia and other hypertensive states must also now be rejected (Spark, 1935).

Approaching the question from a different angle, Fauvet (1931) injected vasopressin into guinea-pigs and reported eclampsia-like lesions in the liver and kidneys, but Ohligmacher (1933) was unable to confirm this. The present research was designed to settle this last question.

### *Experimental methods*

The animals used were healthy female albino rats, weighing from 30-200 g, obtained from Messrs Glaxo. Of these 40 received vasopressin (Pitressin, Parke Davis), the remainder oxytocin (Pitocin) (6 rats) or nothing





LAFAYETTE BENEDICT MENDEL

## LAFAYETTE BENEDICT MENDEL

(February 12, 1872 – December 9, 1935)

On the Sheffield campus at Yale stands a brown stucco mansion, soon to be removed for the extension of the Electrical Engineering Building. This house is fondly remembered by a diminishing number of American biochemists as the laboratory in which Professor Lafayette B. Mendel held his classes and conducted research for almost a third of a century. The office room was restricted; the necessity for employing irregular spaces such as the original parlor and the art gallery, led to make-shift adjustments; and the laboratory equipment, even by the standards of those simpler days, was inadequate. Yet from this laboratory issued not only the record of fundamental research of a high order of excellence, but also several generations of investigators and teachers, most of whom later occupied chairs of biochemistry in widely scattered academic centers in our country. Although in 1923, the Department of Physiological Chemistry was moved to more adequate quarters in the Medical School, the accomplishments in those early years alone are proof of the scientific acumen, the pedagogical genius, and the administrative skill of Doctor Mendel.

The education of Mendel was rather typical of his time; after graduating from Yale in 1891 (he was then 19), he set out to prepare himself for a scientific career. His outlook was profoundly influenced by Chittenden who had established at Yale the first teaching laboratory in physiological chemistry in this country. Mendel became Chittenden's assistant and carried on graduate work which led to the Ph.D. degree in 1893. He remained at Yale as an instructor in the Sheffield Scientific School for two years before deciding to avail himself of the instruction and research opportunities afforded

by European laboratories. He was well equipped intellectually and had become a proficient chemist. He went to Breslau where he studied first with Heidenhain and later with Röhmman. Despite the fact that Mendel was prepared to personally provide material as well as apparatus, permission to work in Heidenhain's laboratory was not given until the new American student exhibited what was to Heidenhain a novelty — a sample of crystallized protein. From the experience in Breslau began Mendel's interest in experimental physiology and the technical approach to it; he always felt that Heidenhain had a great influence upon his subsequent scientific activities. From Breslau Mendel went to Freiburg where for a time he studied chemistry with Baumann.

Upon returning in 1896, Mendel was appointed Assistant Professor in Chittenden's laboratory at Yale. Here began a period of teaching and extraordinary research activity which was to continue for almost 40 years. The early investigations reflect somewhat the interest of his mentor in the chemistry of digestion and absorption, although as early as 1898, in a paper on the nutritive value of fungi, he showed his developing interest in the new science of nutrition. Until about 1910, however, Mendel continued to look into the many paths in the area of gastrointestinal physiology, the beginning of wisdom for anyone interested in nutritional biochemistry. With increasing independence and with the help of numerous graduate students, he examined the enzymatic factors in digestion and the avenues of absorption of various nutrients. An extensive study of the pathway of excretion of certain inorganic salts was made as well as of the intermediary metabolism of purines. In 1905 and 1906 a series of papers on the physiology of the molluscs was published; this activity shows the catholicity of his interest in comparative physiological chemistry upon which he drew so effectively for his delightful lectures. During the following two years (1907 to 1908) several papers appeared dealing with various phases of the chemical physiology of the embryo. Toward the end of this period, studies on the absorption of fat and on the utilization of carbohydrate

and protein were carried out and a long series of experiments on creatine and creatinine was reported. It is of interest that about this time occasional papers were published in the German journals.

Mendel's growing interest in nutrition and his familiarity with the then current methods of experimentation in this field, led him to the conclusion that only through the use of purified experimental diets could definitive information on the nutritive significance of foodstuffs, notably the proteins, be secured. He felt that he needed the assistance of a chemist skilled in the field of protein chemistry for the most effective prosecution of this program of investigation. What were the details of the initial meeting of minds of Osborne and Mendel is unimportant here; it is important that in New Haven, there were these two scholarly men, each in a situation which permitted him to engage in a scientific collaboration under almost ideal conditions, a circumstance to prove extraordinarily fortunate for the progress of science in America. It would not be easy to find two more diverse personalities: Osborne, the shy, retiring savant, and Mendel, the sociable, extrovert scholar with unusually broad interests. However, they possessed one attribute in common, namely, a deep personal and professional regard for the attainments and intellectual honesty of the other. During the almost 20 years of the collaboration, each learned much from the other and each exerted a searching critical influence upon the planning of the experiments as well as on the preparation of the papers, most of which are models of expository writing.

When Osborne and Mendel began their work, one of the most discussed questions in the field of nutrition was the optimal quantity of protein in the diet. Liebig, Moleschott, Voit, Rubner, Hindhede, Chittenden and McCay, among others, had discussed the problems from various points of view with various emphases, and with little unanimity of conclusion. Osborne and Mendel initially set for themselves the task of examining the importance of the quality of dietary protein

in nutritive success or failure, a decision which naturally turned the searchlight of their inquiry upon the amino acids. Aided by a continuing grant from the Carnegie Institution of Washington, the research program was begun at the Connecticut Agricultural Experiment Station in New Haven; at the outset the details of the breeding, housing and feeding of the albino rat were worked out. The observations arising from these important preliminary experiments were published in a monograph in 1911. The addition of this broad research activity to an already heavy program of investigation, teaching and general university and public service, illustrates the astounding capacity for work shown by Mendel at this stage of his career.

Once Osborne and Mendel had established the experimental procedure, a broad plan of study was initiated. The unusually capable staff in Osborne's laboratory contributed to the preparation of purified proteins from various sources as well as to the analytical values for their content of the various amino acids. Papers by Osborne and Mendel began to appear in considerable numbers — 6 in 1912, 10 in 1915–16, 14 in 1917–18, and so on at an average rate of some 8 papers a year between 1911 and 1927! There were studies on the determination of the comparative nutritive value of various purified proteins from cereal grains, other seeds and plant tissues, for growth and maintenance. This inevitably led to the supplementation of some of the deficient proteins with the missing amino acids and to the basic concept that some of the amino acids derived from the hydrolysis of proteins cannot be readily synthesized by the animal body but must be provided *de novo* in the diet. Within the limits of the experimental procedure, protein minima for growth and maintenance were determined and the minimum daily requirement of certain amino acids was suggested.

Following the initial work on purified proteins, each item in the simplified experimental ration was scrutinized. It was in connection with the examination of the needs of the rat

for inorganic salts that Osborne and Mendel became convinced that in natural foods there were present nutritionally indispensable factors for whose detection and quantitative estimation, methods were then non-existent. Thus, as a result of their demonstration that a mixture of pure salts similar to those found in milk, failed to support nutritive success as did their "protein-free milk" used to provide the ash constituents in their early diets, they were receptive to the suggestion that an indispensable water-soluble factor (vitamin B) was present in milk. Later, a study of the lipid component of the diet, brought out the fact that certain natural fats possess nutritive virtues which are lacking in others, an observation marking their discovery of vitamin A. The natural distribution of this factor and its chemical character and behavior also received attention.

In the course of the evaluation of the carbohydrate portion of the diet, Osborne and Mendel experimented with diets, the components of which were present in extreme proportions. One of the by-products of this line of research was their interest in the response of the kidney to diets unusually rich in protein. The hypertrophic response of the kidney, however, was but one aspect of the larger question of growth which early elicited their interest. Whereas the previous concept of growth was that of an inherited capacity which must act at the proper time (youth) to exert its influence, Osborne and Mendel showed that growth can occur at any time in the life of an animal, once the chemical environment (nutrition) is adequate, other factors being optimal.

The last contribution published by Osborne and Mendel was a summary of their work on growth; after a fruitful period of some 20 years, this scientific partnership came to an end with the death of Osborne in 1929. The work of Osborne and Mendel gave direction to the development of nutritional biochemistry in this country: subsequent extension of the field of essential amino acids, discoveries based on the use of fat-free diets and the extended differentiation between the vitamins, are expansions of our knowledge of nutrition

whose point of departure is the use of experimental rations composed of known purified components.

As stated before, Mendel supervised investigations on a wide variety of topics in chemical physiology in his own laboratory at Yale during and after his work with Osborne at the Experiment Station. The metabolism of calcium and magnesium, the physiology of absorption, transport and secretion, the metabolism of the pyrimidines, non-specific protein reactions, the regulation of blood volume, carbohydrate metabolism, the physiology and distribution of the vitamins, are some of the topics to which he added significant knowledge through collaboration with graduate students. The relation of the chemical character of dietary fat to that of body fat was one of the themes which appealed especially to Professor Mendel and he gave it his enthusiastic attention.

Mendel was a prolific writer; along with his diverse program of investigation he wrote many reviews and editorials on the relation of the growing science of nutrition to medicine, and on the influence of nutritional research in the national economy. Although there are some 340 papers attributed to him, this number is by no means an index of the number of investigations which he directed, for with characteristic generosity, the inclusion of the "Professor's" name on a paper arising from collaboration with a student was left to the choice of the junior partner. Doctor Mendel published three books: "Childhood and Growth," (1906), "Changes in the Food Supply and their Relation to Nutrition," (1916), and "Nutrition, the Chemistry of Life," (1923). He wrote fluently in an accurate and cogent manner and the first draft of his manuscripts rarely required revision.

Mendel's laboratory was an active center of both undergraduate and graduate instruction and he is affectionately remembered by the large number of his students as an inspiring teacher. Early in his career his students were largely undergraduates; in many of these he aroused interest in the new field of physiological chemistry and led them to realize the opportunities in medicine, bacteriology, public health and

## BIOGRAPHY

physiological chemistry itself, stimulating advanced study in these fields. Mendel was systematic and in this attitude lay his strength. He believed that the investigator should do his own work effectively and to this end he would answer to a classroom question or the next report be given not only accurately, but also said, in elegant English. He himself was a speaker and ranged widely for illustration. His demonstrations were carefully planned with finesse, always accompanied by lucid explanation of the inevitable uncertainty of animal experiments. With its ugly head, the situation was usually cleared from his past experience or by bits of observation. He strove to inculcate a respect for skilled work. He permitted no liberties with accuracy on the part of the student. A confirmed experimenter, extremely sensitive to lack of care in handling animals, he insisted that adequate anesthesia and general routine in the demonstration or experiment.

Graduate students began to come to him in his career; his extraordinarily broad knowledge in physiology and biological chemistry suggested a great range of topics worthy of study. He frequently assigning a student to a definite analytical method, Mendel was not without a sense of methodology, for he frequently said, "and I will give you a problem." His teaching was function, approached by the physiological methods then current and this dynamic factor in attracting graduate students was literally guided through the intricacies of "Professor's" own hand; as time went on of a growing maturity in his own science.



able to endow them with a benign but liberal independence in scientific thinking. Graham Lusk wrote of Mendel as a teacher:

“He has been the guide, philosopher and friend to many young men and women; he has encouraged them to walk by themselves when they were able to stand alone; and he has given wise counsel in times of difficulty. Herein he has shown himself as one of the great teachers of his time.”

Probably no part of Professor Mendel's teaching program will be more pleasantly recalled by the participants than his seminar. These stimulating weekly sessions of informal discussion by both the “Professor” and the students, still seem to the writer to be an unequalled teaching device for the advanced student with respect to contact with the problems and the progress in as broad a field as is physiological chemistry.

Richly endowed with factual knowledge, with an engaging personality, and with a deep interest in his students, he gave serious consideration to the methods of pedagogy and to the dignity of the profession of teaching. The prepared mind and a willingness to work hard were for him evidence enough that the student merited his guidance. He almost literally looked upon his graduate students as his “laboratory family” and took great satisfaction in the distinguished subsequent careers of many of them.

Mendel's outstanding position in American science and his gift for administration early led the University to make use of his talents. A member of the faculty for 43 years, he served variously on the governing boards of the Sheffield Scientific School, the Graduate School, the University Library Committee, and the Board of Permanent Officers of the Medical School. A keen judge of men, it was natural that he should have served at various times on fellowship committees and on the Admissions Board of the Undergraduate Schools. Appreciation of Mendel's University service is shown by his appointment in 1921 to one of the first Sterling Professorships.

Outside the University, Mendel's advice and assistance was sought in the fields of nutrition, public health, and medicine. He was an official member of several international congresses. He recognized the importance of organized effort of scientific groups; thus he served as secretary of the American Physiological Society for several of its early years and was active in the formation of the American Society of Biological Chemists in 1906, serving this group in the various offices. He was the first President of the American Institute of Nutrition and helped guide the Editorial policy of the Journal in its early years. He enjoyed scientific editorial work and performed important service of this nature for *Chemical Abstracts*, the *Journal of Biological Chemistry*, *Scientific Monographs* of the American Chemical Society, and the *Journal of the American Medical Association*. In view of the meticulous attention given by him to these extra duties, one marvels again at Mendel's enormous capacity for work.

Doctor Mendel's career spanned the period of the development of nutrition and he was looked upon as one of the leading protagonists of this new area in the field of physiological chemistry. His prolific contributions to the scientific literature and his effectiveness as a speaker, led to many such engagements. In 1906 he gave one of the first Harvey Lectures and again in 1914 he discussed the chemical aspects of growth before that society. He spoke on one of the Sigma Xi lecture tours and gave the 1914 Herter Lectures at University and Bellevue Hospital Medical College in New York. Nine years later he gave the Hitchcock Lectures at the University of California. In 1930 he lectured on the Schiff Foundation at Cornell University and was Cutter Lecturer on Preventive Medicine at the Harvard Medical School. During all of this time he was in demand as a speaker to civic and scientific groups; indeed, he can be looked upon as one of the early outstanding forces in the popularization of science.

It seems inevitable that Dr. Mendel's broad interest in physiology, toxicology and chemistry should have brought him into more or less close contact with medicine. For the

greater part of his professional life, he advised on national medical problems, wrote for medical journals, and spoke to medical audiences. As he took part in the development of nutrition he became convinced, and so urged, that it be looked upon as an exceedingly important factor in preventive medicine. He was frequently consulted by the practitioner regarding clinical problems; the discussion usually revolved about normal physiology and chemistry upon the basis of which guidance to the solution of the problem was usually given.

During his lifetime many honors came to Dr. Mendel. He was proud to have been a charter member of the Yale Chapter of Sigma Xi. Later, honorary degrees were conferred by the University of Michigan, Rutgers University, and Western Reserve University. He was long a member of the National Academy of Sciences and of the American Philosophical Society. In 1929 he was elected to membership in the Societe de Biologie in Paris and became a member of the American Academy of Arts and Sciences a year later. His academic service was recognized by a gold medal given by the American Institute of Chemists and a year before his death, the Chemists' Club of New York conferred upon him the Conne Medal for outstanding chemical service to medicine. When he was 60, his friends, students, and professional associates presented him with his portrait and for the same occasion, there was published an Anniversary Number of the *Yale Journal of Biology and Medicine* containing articles by some of his former pupils. While he valued these various honors, he prized most of all the successes of his many former students.

In Dr. Mendel's death on December 9, 1935, at the age of 63, there passed not only one of the pioneers in the science of nutrition, but also a gentle friend to many whose lives were enriched by contact with him.

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# STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT TREATMENT<sup>1</sup>

## IV. ETHYL ESTERS OF HEAT-POLYMERIZED LINSEED, SOYBEAN AND SUNFLOWER SEED OILS

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(Received for publication March 31, 1956)

### INTRODUCTION

The literature on the effects of polymerization temperature on the nutritive value of edible oils has been reviewed up until 1952 by Crampton et al. ('53). Since then Frahm et al. ('53) have reported deleterious effects of heat-polymerized whale oil when it is fed to mice. Raju and Ragagopalan ('55) have reported the results of feeding rats with diets containing 15% of peanut or sesame or cocoanut oil which had been heated at 270°C. in open pans in contact with air. The effects included depression of live weight gain, decrease in food efficiency and increases in liver weight as percentage of body weight and in percentage of liver fat. However, although the temperature used by Raju and Ragagopalan suggests that there was polymerization, the experiments of these workers are not comparable with those carried out in our laboratories where the oils were heated in a current of CO<sub>2</sub>. Kaunitz et al. ('55) have found that cottonseed oil heated and aerated at 90 to

<sup>1</sup>Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Province of Quebec, Canada. Journal Series no. 394.

95°C. for periods of up to 300 hours became injurious to rats. Incorporation of fresh oil gave a degree of protection against some of the deleterious effects; peroxides were considered not likely to be responsible for the ill effects. The conditions of heating differed greatly from those used in our laboratories.

In a previous paper (Crampton et al., '53) we have reported a study of the nutritional properties of certain fractions prepared from the ethyl esters of heat-polymerized linseed oil by distillation and urea adduct formation. The preparation and the designations of the different fractions are described in this previous paper, as are also the diets and plan of the feeding trials. For convenience of reference the flow sheet for fractionation is reproduced in figure 1.

It should be pointed out here that polymerization of triglyceride oils is now known definitely to include formation of significant amounts of trimeric, and even of some higher polymeric acyl radicals, as well as of dimeric acyl radicals (Paschke and Wheeler, '54). In the present paper, therefore, fraction 6 is designated "polymer" rather than "dimer."

From this earlier work it seemed reasonable to suppose that heated linseed oil was nutritionally injurious, firstly, because of the presence of polymerized material that is poorly absorbed, if at all, and secondly, because of the presence of monomeric acyl radicals incapable of forming urea adducts by reason of some structural feature, possibly a cyclization. In this connection it is noteworthy that Paschke and Wheeler ('55) have now demonstrated the formation of a cyclic monomer during heat polymerization of methyl eleostearate and have shown that this cyclic monomer is mainly an ortho-disubstituted cyclohexadiene.

The question that next presented itself was the degree to which formation of non-adduct-forming monomeric material could be related to the fatty acid composition of the original oil itself. Accordingly, in 1953-'54 further rat feeding trials were carried out involving 24 lots of 10 animals each in order to study the nutritional value of similar fractions prepared from soybean oil and sunflower seed oil. Soybean oil was

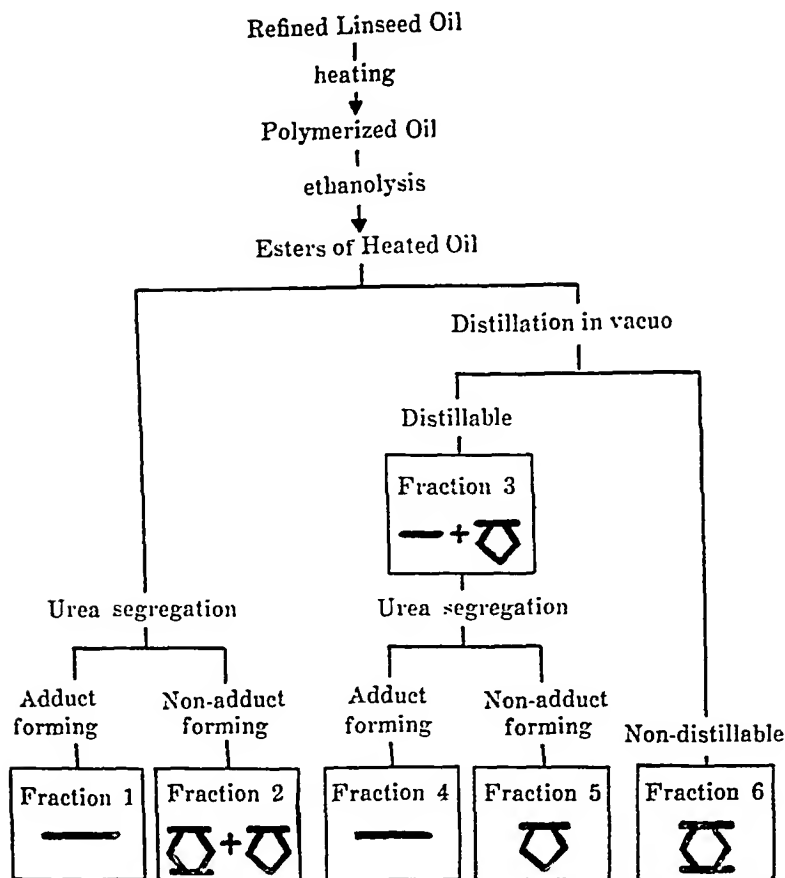


Fig. 1 Flow sheet illustrating preparation of fractions of esters of heated linseed oil used in feeding trials.

chosen because it is a food oil that contains some linolenic acid. Sunflower seed oil was chosen because of its high linoleic acid content and negligible linolenic acid content. Typical data for the fatty acid composition of the three oils are summarized in table 1.

Preliminary experiments with soybean and sunflower seed oils showed that non-adduct-forming distillable ester (NAFD) fractions could be obtained from both oils using the same polymerization temperature (275°C.) and heating in a current of CO<sub>2</sub>. It was necessary to heat these oils for longer times

TABLE 1

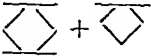



*Fatty acid composition by weight of linseed, soybean and sunflower seed oils*

	SATURATED			UNSATURATED		
	C <sub>16</sub>	C <sub>18</sub>	C <sub>20</sub>	Oleic	Linoleic	Linolenic
	%	%	%	%	%	%
Linseed N. American (I No. 179.8) <sup>1</sup>	6.3	2.5	0.5	19.0	24.1	47.4
Soybean (I No. 132.5) <sup>2</sup>	10.6	2.4	2.4	23.5	51.2	8.5
Sunflower seed <sup>3</sup>	6.4	1.3	4.0	21.3	66.2	.

<sup>1</sup> Rose and Jamieson ('41), cited by Bailey ('52).<sup>2</sup> Hilditch, Meara and Holmberg ('47), cited by Bailey ('52).<sup>3</sup> Barker, Crossley and Hilditch ('50), cited by Bailey ('52).

TABLE 2

*Yields of fractions of ethyl esters used in feeding trials*

FRACTION		YIELD AS PER CENT OF TOTAL ETHYL ESTERS OF HEATED OIL		
		Linseed <sup>1</sup> (12 hr. at 275°C.)	Soybean (24 hr. at 275°C.)	Sunflower seed (26 hr. at 275°C.)
1. Adduct-forming fraction of total esters	—	46 (293) <sup>2</sup>	63 (296)	65 ( )
2. Non-adduct-forming fraction of total esters		54 (472)	37 (415)	35 ( )
3. "Distillable" esters	— + 	60 (294)	74 (296)	75 (303)
4. Adduct-forming fraction of distillable esters	—	49 (293)	64 (294)	65 (295)
5. Non-adduct-forming fraction of distillable esters		11 (300)	10 (294)	10 (296)
6. Non-distillable esters		40 (550)	26 (415)	25 (613)

<sup>1</sup> Data for linseed oil quoted from Crampton et al. ('53).<sup>2</sup> Figures in parentheses are cryoscopic mean molecular weights.

than linseed oil in order to attain reasonable yields of this fraction. The times used for bulk preparation of fractions are given in table 2, together with approximate yields of the various fractions. The data for linseed oil are quoted from Crampton et al. ('53).

## RESULTS






Tables 3 to 7 summarize the results of the feeding experiments with soybean oil and sunflower seed oil, the data for linseed oil being cited from Crampton et al. ('53). In the discussion that follows, the data for linseed oil are considered along with those for the two other oils; the latter sets of data are here reported for the first time.

*Survival of rats.* The percentages of the rats surviving the 28-day feeding are shown in table 3.

TABLE 3  
*Comparison of the nutritional effects of ethyl esters of linseed,  
soybean and sunflower seed oils*

(Ten rats per lot)

Percentage of rats surviving 28-day test

DIET	ESTER FRACTION	LINSEED		SOYBEAN		SUNFLOWER SEED	
		20%	10%	20%	10%	20%	10%
1	—	100	100	100	100	100	100
2	 + 	20	100	0 <sup>1</sup>	100	50 <sup>2</sup>	50 <sup>2</sup>
3	— + 	90	100	100	100	100	100
4	—	100	100	100	100	100	100
5		0	0	100	80 <sup>3</sup>	100	100
6		70	100	0 <sup>1</sup>	10 <sup>4</sup>	100	100

<sup>1</sup> All animals removed after 10 days because of diarrhea and extreme viscosity of feces.

<sup>2</sup> Five animals removed because of diarrhea.

<sup>3</sup> Remaining rats in poor condition.

<sup>4</sup> Some diarrhea.








The only deaths recorded were in lots where the diets contained either non-adduct-forming monomers or dimeric or higher polymers, with the former displaying the greater toxicity. Also, in every lot receiving esters of polymeric acids there was diarrhea and the feces were varnish-like. This material was so sticky that at morning inspection the feet and tails were often found to be inseparable without washing. In some cases the animals were stuck to the wire floor of their cages. The diets, however, did not appear to be toxic in the usual sense of the term.

The ester fraction which consisted entirely of urea adduct-forming monomers had no harmful effect on survival. The one death in lot 3 may have been due to the "cyclic" monomers of linseed oil also present, since all rats died in lot 5 even where the diet contained but 10% of such material.

*Gain of rats.* The gain figures shown in table 4 are not directly comparable as between oils. For each ester source, however, figures are strictly comparable as between diets and between levels.

TABLE 4  
Live weight gains—28 days

DIET	ESTER FRACTION	LINSEED		SOYBEAN		SUNFLOWER SEED	
		20%	10%	20%	10%	20%	10%
		gm	gm	gm	gm	gm	gm
1	—	101	107	93	99	143	150
2	 + 	32	39	..	63	107	150
3	— + 	4	66	83	101	162	162
4	—	57	108	91	99	151	159
5		.	.	16	36	97	142
6		13	77	..	69	90	143
Least significant difference ( $P = 0.05$ )		23	23	10	10	20	20

The results leave little doubt that esters of both "cyclic" monomeric and polymeric acids from the heat polymerization of these three oils are undesirable components of rat diets. However, there would seem to be a difference in the degree of toxicity of the non-adduct-forming distillable fractions, insofar as that from sunflower seed oil was less damaging than those derived from flaxseed or soybean oil. These oils differ chiefly in their contents of the trienoic linolenic acid and there is a temptation to ascribe to this fatty acid the origin of the toxic "cyclic" monomers. Such an ascription requires an assumption that some non-adduct-forming material arises on heat polymerization from some fatty acid other than linolenic and perhaps also that this is a less toxic material than that formed from trienoic fatty acids.

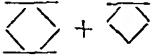




*Food intake.* Fractions 1 and 4 were equally readily eaten, except at the 20% level of the linseed oil fractions. The polymer fraction 6 was practically equally acceptable, except at the 20% level of the linseed oil fraction; this is remarkable in view of the unattractive varnish-like nature of the materials in question. The "cyclic" monomer fraction (fraction 5) was less readily eaten than the other fractions; and here it is to be noted that these fractions were either colorless or very pale, bland oils. Fractions 2 and 3, containing some "cyclic" monomer, were as readily accepted as fractions 1 and 6, in the case of the soybean and sunflower seed oil fractions, but less readily in the case of the linseed fraction. On the whole, these results suggest that the only fraction that was definitely poorly acceptable was fraction 5.

*Digestibility of the oils.* There was reasonably clear evidence that the non-adduct-forming polymers were poorly digestible. This was predictable from the abnormal feces. It is probable that low digestibility was a major causal factor in the slower gain of the animals fed this oil fraction. The digestibility of other fractions was above 90%.

*Efficiency of utilization of dietary calories (gain per 1000 digested calories).* The data suggest that, in general, the rats used that portion of the calories which they absorbed about

equally well excepting for the "cyclic polymers." In the case of the linseed and soybean oils, the digested polymeric esters were in some cases as efficient as the straight-chain materials, but with sunflower seed oil this fraction was as unsatisfactory as the cyclic monomers (table 5).

TABLE 5  
*Gains per 1000 digested calories*

DIET	ESTER FRACTION	LINSEED		SOYBEAN		SUNFLOWER SEED	
		20%	10%	20%	10%	20%	10%
1	—	gm 61	gm 61	gm 99	gm 99	gm 82	gm 89
2	 + 	9	50	.	88	69	101
3	— + 	43	63	92	104	95	90
4	—	54	62	99	106	92	87
5		..	..	85	85	77	93
6		61	56	..	88	65	84
Least significant difference ( $P = 0.05$ )		16	16	10	10	14	14

#### DISCUSSION

Certain characteristics of the non-adduct-forming distillable ester fractions from the three oils are presented in table 6. The order of decreasing injuriousness was also the order of decreasing iodine value and refractive index, while the cryoscopic mean molecular weights were, for practical purposes, the same and corresponded to a preponderance of  $C_{18}$  acids. The most marked difference between the three NAFD fractions was in respect to their behaviour on alkali isomerization (fig. 2). Linseed NAFD displayed a relatively low absorption at 233  $m\mu$ , while both soybean NAFD and sunflower seed

TABLE 6

*Iodine values and refractive indices ( $n_D^{20}$ ) of fractions of ethyl esters of linseed, soybean and sunflower seed oils*

FRACTION		LINSEED	SOYBEAN	SUNFLOWER SEED
1. Adduct-forming fraction of total esters	Iodine no.	118.2	99	..
	$n_D^{20}$	1.45345	1.45302	..
2. Non-adduct-forming fraction of total esters	Iodine no.	162.7	125	..
	$n_D^{20}$	1.47561	1.46998	..
3. "Distillable" esters	Iodine no.	130.1	106	110
	$n_D^{20}$	1.45684	..	..
4. Adduct-forming fraction of "distillable" esters	Iodine no.	124.8	92	107
	$n_D^{20}$	1.45494	1.44935	1.45254
5. Non-adduct-forming fraction of "distillable" esters	Iodine no.	170.7	143	130
	$n_D^{20}$	1.46986	1.47001	1.45671
6. "Non-distillable" esters	Iodine no.	159.9	114	106
	$n_D^{20}$	1.48017	..	1.47655

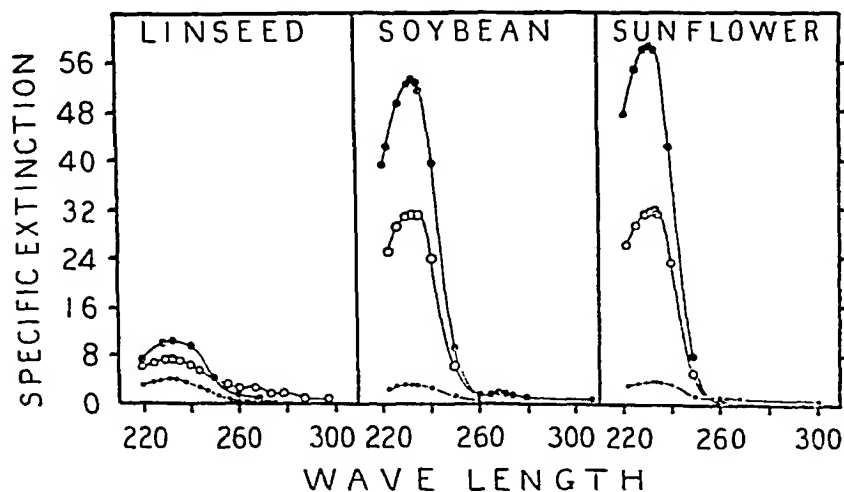


Fig. 2 Ultraviolet absorption spectra of non-adduct-forming distillable fractions of the esters of heat polymerized linseed, soybean and sunflower seed oils.

- unisomerized.
- alkali-isomerized for 25 minutes.
- alkali-isomerized for 6 hours.

NAFD displayed a high proportion of difficultly conjugable diene unsaturation, as shown by the increase in absorption at 233  $\mu$  on extending the isomerization time from 25 minutes to 6 hours. This increase could be explained by the presence of much *cis-trans* acid (Jackson, Paschke, Tolberg, Boyd and Wheeler, '52). The results with linseed oil suggest a much lower proportion of difficultly conjugable *cis-trans* isomer. In point of fact, oxidation with permanganate by Bertram's method showed that linseed NAFFD contained less than 2% of saturated material, and the spectrometric data and iodine value suggest that linseed NAFFD contained a high proportion of non-conjugable diene, and perhaps as much as 80%. But we are not yet in a position to state what feature of the chemical construction is responsible for the failure to form an urea adduct. It is, of course, possible that this feature may be related to the nutritional defectiveness of the fraction; at the same time it must be borne in mind that failure to form urea adducts may result from a variety of structural features.

#### SUMMARY

The non-adduct-forming fraction (NAFFD) of the distillable esters from heated soybean oil was toxic, though to a lesser degree than that from the comparable fraction obtained from linseed oil. The NAFFD from heated sunflower seed oil, however, was only slightly injurious to the rats.

The adduct-forming fractions from both the heated soybean oil and the heated sunflower seed oil were nutritionally harmless.

The chief chemical difference between the NAFFD fractions from the three heated oils was in respect to their behaviour on alkali isomerization. The NAFFD from heated linseed oil displayed relatively little increase of its absorbance at 233  $\mu$ , whereas the results for the soybean and sunflower seed oils suggested the presence of high proportions of difficultly conjugable diene unsaturation.

These results suggest that the non-adduct-forming fraction of the distillable esters of heated linseed may contain a high

proportion of non-conjugable diene *cis*-isomers, possibly of cyclic structure.

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#### APPENDIX

Preparation of ethyl esters of polymerized soybean, sunflower seed and linseed oils.

Raw solvent-extracted soybean oil (Victory Mills Limited, Toronto) was alkali-refined in batches of 1.5 kg with 3.6% 20° Baumé sodium hydroxide, washed and dried with sodium sulphate. The correct amount of alkali was calculated from the acid value and the tables given by Bailey ('51). Batches of 500 gm of the oil were then bleached with 2% Super Filtrol, filtered and polymerized at 275°C. for 20 hours under a stream of CO<sub>2</sub>. From that point the preparation was as described by Crampton et al. ('55).

Raw sunflower seed oil (Co-operative Vegetable Oils Limited, Altona, Man.) was handled in the same fashion, except that a heating time of 26 hours was selected for the polymerization. Preliminary experiments showed that there was not any appreciable formation of NAFD fraction until this oil had been heated for 20 hours. The subsequent steps in the preparation of ester fractions were performed as for the linseed and soybean oils.

The yields and mean molecular weights of the various fractions are given above in table 2. The corresponding iodine numbers and refractive indices are reported in table 6.

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# DEVELOPMENT OF FATTY LIVERS DURING LACTATION OF RATS FED AMINO ACID RATIONS<sup>1</sup>

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During earlier studies on the reproduction of rats fed protein-free amino acid rations, it was noted (Schultze, '55) that after completion of lactation the livers of rats were greatly enlarged, pale colored, mottled and friable. Since this condition was not observed in non-lactating animals of similar age fed the same rations, it appeared that fatty livers had been induced by the stress of lactation. This condition was investigated in conjunction with extended studies (Schultze, '56) of the adequacy of protein-free amino acid rations for reproduction and lactation. Moreover, since the literature does not appear to contain a record of previous systematic observations on this point, it was necessary to investigate the effect of pregnancy and lactation on the lipid content of livers of rats fed adequate rations composed largely of natural products. The results of this work are summarized in this paper.

## EXPERIMENTAL

*Rations.* The rations contained the ingredients listed in table 1. Amino acid mixtures I and II which contained 10 and 16 amino acids respectively had the same composition as previously described (Schultze, '55) except that the isoleucine

<sup>1</sup> Paper no. 3513, Scientific Journal Series, Minnesota Agricultural Experiment Station.



was from a different source.<sup>2</sup> The rolled oats — casein ration OC<sub>4</sub> differed from similar rations previously described (Gander and Schultze, '55) in that unpurified commercial casein was substituted for the leached casein prepared in the laboratory. Such a ration can support very heavy lactation (Schultze, '54) and simultaneous weight gains of mothers. The rations were mixed at least once a week and stored at 4°C.

TABLE 1  
*Composition of rations*

COMPONENT	R A T I O N					
	AA <sub>4</sub>	AA <sub>17</sub>	AA <sub>10</sub>	AA <sub>17</sub>	AA <sub>10</sub>	OC <sub>4</sub>
Amino acid mixture I, <sup>1</sup> gm	159.4	....	....	....	....	....
Amino acid mixture II, <sup>1</sup> gm		122.3	183.4	122.3	244.5	....
Diammonium citrate, gm	51.6	.	.	.	.	.
Casein, commercial, gm		..	.	.	..	65.0
Rolled oats, ground, gm			.	.	.	840.0
Sucrose + B vitamin mixture I, <sup>2</sup> gm	20.0	20.0	20.0	20.0	20.0	20.0
Salt mixture IV, <sup>3</sup> gm	40.0	40.0	40.0	40.0	40.0	30.0
Sucrose, gm	699.0	787.7	726.6	687.7	665.5	11.7
Hydrogenated vegetable oil, <sup>4</sup> gm			.	100.0	.	..
Wheat germ oil, gm	20.0	20.0	20.0	20.0	20.0	20.0
Corn oil + vitamins A and D, <sup>1</sup> gm	10.0	10.0	10.0	10.0	10.0	10.0
DL-Methionine, gm	...	.	.	.	..	3.3
Pyridoxine hydrochloride, mg	5		.		..	....
Total grams	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0

<sup>1</sup> Schultze, '55.

<sup>2</sup> Gander and Schultze, '55.

<sup>3</sup> Schultze, '50.

<sup>4</sup> Crisco.

*Animals.* All rats were from our black strain of line 3 which had been used in earlier studies (Schultze, '55). They were housed in groups of 5 in cages provided with raised screens except shortly before parturition and during lactation, when a cage, bedded with clean wood shavings, was provided for each animal.

*Rats fed the rolled oats — casein ration.* Littermate female rats, three weeks old, whose mothers had consumed the same

<sup>2</sup> Nutritional Biochemicals Corporation, Cleveland, Ohio.

ration were allotted at random to 7 groups and fed ration OC<sub>4</sub> throughout the experiment. The rats in groups 1a, 1b, and 1c, table 2, were not bred; all others were bred at 10 weeks of age. The rats in group 1a were killed when their littermates were bred; those in group 1b at parturition of their littermates, and those of group 1c at the end of a three weeks period of lactation of their littermates. The rats in group 2 were killed within 7 hours after birth of their young. Within 12 hours after parturition, the number of young nursed by rats in group 3a was reduced to two; in group 3b to 6, and in group 3c it was increased to 12, where required, by the addition of young from groups 3a or 3b. When any nurslings died, they were replaced by rats of about the same age obtained from litters which were raised for this purpose. Thus it was possible to compare the lipid content of the livers of rats at the end of pregnancy or at the end of a lactation period of varying degree of intensity with that of non-lactating littermates of the same age. The rats in groups 3a, 3b, and 3c were killed after a period of lactation of 21 days.

*Rats fed protein-free amino acid rations.* These animals were offspring from mothers fed the same or similar protein-free rations. With the exception of group 8, table 3, fed ration AA<sub>17</sub>, each group was fed the same ration from weaning until killed. Group 8 was transferred from ration AA<sub>15</sub> to AA<sub>17</sub> after the second litters had been weaned. All animals in groups 2, 5, 7, and 9, table 3, were killed on the 28th day of their second lactation; those in group 8 on the 28th day of their third lactation; those in groups 3 and 6 were killed 14 days after completion of their second lactation period of 28 days. The size of the litters was not changed by addition or removal of young after parturition and the mean number of young nursed for 28 days ranged from 6.0 to 7.3 per mother in all groups except 1, 4 and 10. The latter three included a few animals which lost their litters through abortion or shortly after birth, i.e. all rats in these groups were subjected to no stress

of lactation or to a very light one. These rats were killed when their litters died or after lactating for 28 days.

*Determination of liver lipids.* The livers were removed from the decapitated rats, weighed and partially dried at 60° C. The whole liver was then macerated and representative samples were used for analysis of residual moisture and of lipids by a procedure previously described (Hedin and Schultze, '55).

TABLE 2  
*Effect of pregnancy and lactation on liver lipids<sup>1</sup>*

GROUP	REPRO- DUCITIVE STATUS <sup>2</sup>	BODY WT.  gm	L I V E R		
			Per cent of body weight	Dry weight  % of fresh	Lipids  % of dry weight
1a	N.P.	171 ± 4.7 <sup>3</sup>	3.88 ± 0.09	29.6 ± 0.37	21.6 ± 0.60
1b	N.P.	205 ± 6.2	3.62 ± 0.26	29.0 ± 0.78	23.0 ± 0.85
1c	N.P.	207 ± 3.8	3.42 ± 0.07	28.9 ± 0.33	21.5 ± 0.68
2	P	221 ± 5.5	3.89 ± 0.05	27.4 ± 0.27	24.7 ± 0.58
3a	N <sub>2</sub>	247 ± 6.5	3.85 ± 0.10	28.2 ± 0.59	20.8 ± 1.05
3b	N <sub>6</sub>	235 ± 3.4	4.92 ± 0.19	28.9 ± 0.30	20.3 ± 0.33
3c	N <sub>12</sub>	236 ± 3.6	4.88 ± 0.10	28.0 ± 0.25	21.4 ± 0.92

<sup>1</sup> Means of 10 animals except group 3c which comprised 14 animals; all rats fed ration OC.

<sup>2</sup> N.P. = not pregnant; P = killed after parturition; N<sub>2</sub>, N<sub>6</sub>, N<sub>12</sub> = killed after nursing 2, 6, 12 young respectively for 21 days.

<sup>3</sup> Standard error of the mean.

## RESULTS AND DISCUSSION

*Lipid content of livers of rats fed a natural ration.* The results obtained with the rolled oats — casein ration are summarized in table 2. Comparison of the data from groups 1a, 1b and 2 indicate that during pregnancy there was only a slight, statistically non-significant ( $P = > 0.10$ ) increase in the lipid content of the livers. During lactation, however, the lipid content of the livers remained in the range of values found in non-lactating littermates of the same age (compare groups 3a, 3b, and 3c with group 1c). If it is assumed that the lactating rats at parturition had the same content of liver lipids as their littermates (group 2) which were killed shortly

after parturition, there was in all three lactating groups a statistically significant decrease in concentration of liver lipids; (comparing groups 3a and 3b with group 2,  $P = < 0.01$ ; comparing group 3c with group 2,  $P = < 0.02$ ). Evidently even the heaviest stress of lactation, when supported by an adequate diet did not induce fatty livers. In the mothers which nursed 6 or 12 young, there was, however, a significant ( $P = < 0.01$ ) increase in the mass of the liver, not accounted for by increased proportions of water and lipid. The livers of all groups had essentially the same concentration of solids. Even during the heaviest lactation the mothers gained weight. Poo et al. ('39) observed that the weight of the livers of rats increased during pregnancy but not during lactation whereas Field et al. ('42) found an increase both in pregnancy and lactation. Beauvallet ('53) observed fatty livers in a small proportion of rats killed at the end of lactation. Beaton et al. ('54) found that the absolute size of the liver increased during pregnancy of rats.

The amount and composition of the diet consumed can have a profound effect on the liver fat in pregnancy. Thus, Ferguson ('54) observed that pregnant sheep fed on a high plane of nutrition had normal livers whereas those on a low plane of nutrition developed fatty livers, associated sometimes with toxemia of pregnancy (Parry, '54). Himsworth ('47) found that the stress of pregnancy can induce gross fatty infiltration of livers of rats fed diets which contain just sufficient choline and casein to prevent fat accumulation in non-pregnant animals. Instances of fatty and enlarged livers associated with pregnancy have also been observed among Africans subsisting on diets deficient in protein (Woodruff, '51).

*Lipid content of livers of rats fed protein-free amino acid rations.* Table 3 presents a summary of the results obtained with 5 different protein-free rations. In young growing rats, rations AA<sub>4</sub> and AA<sub>15</sub> produced a temporary accumulation of liver fat<sup>3</sup> but as the animals became older the lipid con-

<sup>3</sup> Hallanger and Schultze, unpublished data.

TABLE 3  
*Effect of lactation on lipid content of livers of rats fed amino acid rations*

GROUP	MATION	NUMBER OF RATS	YOUNG NURSED PER LITTER	LIVER REMOVED ON DAY	BODY WEIGHT gm	L I V E R		
						Per cent of body weight	Dry weight	Lipids
						% of fresh	% of dry weight	
1	AA <sub>4</sub>	7	0-2	<sup>1</sup>	177 ± 9.5	4.55 ± 0.22	27.6 ± 1.43	25.5 ± 1.09
2	AA <sub>4</sub>	19	> 3	P + 28 <sup>2</sup>	164 ± 4.1	11.27 ± 0.61	44.6 ± 2.2	66.1 ± 1.07
3	AA <sub>4</sub>	8	> 3	P + 42 <sup>3</sup>	193 ± 5.1	5.86 ± 0.40	37.6 ± 1.25	46.3 ± 3.71
4	AA <sub>13</sub>	5	0-2	<sup>1</sup>	223 ± 1.8	4.23 ± 0.06	26.9 ± 1.46	29.3 ± 2.41
5	AA <sub>13</sub>	16	> 3	P + 28	177 ± 4.9	5.89 ± 0.17	34.0 ± 0.69	41.9 ± 2.23
6	AA <sub>13</sub>	10	> 3	P + 42	198 ± 6.8	4.08 ± 0.09	30.5 ± 0.43	29.4 ± 1.18
7	AA <sub>19</sub>	9	> 3	P + 28	212 ± 4.3	7.08 ± 0.27	39.5 ± 1.71	55.8 ± 2.94
8	AA <sub>17</sub>	6	> 3	P + 28	228 ± 12.0	6.37 ± 0.46	36.9 ± 1.5	45.8 ± 3.5
9	AA <sub>19</sub>	8	> 3	P + 28	220 ± 14.5	6.39 ± 0.37	36.2 ± 2.2	49.9 ± 3.7
10	AA <sub>19</sub>	3	0-2	<sup>1</sup>	226	3.94	27.7	25.3

<sup>1</sup> After abortion, death of litter or 28 days after birth of 1 to 2 young.

<sup>2</sup> P + 28 = 28th day after parturition, the day when young were weaned.

<sup>3</sup> P + 42 = 42nd day after parturition, 14 days after young were weaned.

centration was only slightly higher than that found in rats fed an adequate ration. The results obtained with groups 1, 4 and 10, table 3, which included rats that aborted, lost their young shortly after birth, or nursed only one or two young indicate that during pregnancy a marked increase of the lipid content of the liver had not occurred. In contrast to this, however, all groups which were subjected to a strong stress of lactation and then killed, showed an increase in the size and particularly in the lipid content of the livers. When the diet contained only 10 amino acids (group 2), this increase was particularly striking even though the amino acids were furnished at a higher level than in ration AA<sub>15</sub> containing 16 amino acids (group 5). An increase in the fat and energy content of the ration did not prevent fatty infiltration of the livers (compare groups 5 and 8) while an increase in the amino acid content from 12.2 (group 5) to 18.3% (group 7) increased the relative size of the livers and their lipid content. A further increase of the amino acid content of the ration to 24.4% failed to prevent enlargement or fatty infiltration of the livers (group 9). With these diets the hypertrophy and fatty infiltration of the liver is clearly the result of the stress of lactation because within two weeks after weaning, the size and fat content of the liver decreased towards normal levels as indicated by comparing groups 2 and 5 with groups 3 and 6 respectively. Coincident with the increase in fat content of the livers there was also an increase in the absolute and relative amounts of non-fat solids, presumably protein. The moisture content of the livers decreased, however, with increasing liver fat. The temporary hypertrophy and fatty infiltration of the livers apparently did not inflict permanent damage because many rats fed the same rations had several successive pregnancies and lactations and their young had a very low rate of mortality (Schultze, '56).

The reason for the development of fatty livers during lactation is not clear at present. Doubling of the methionine content of the ration did not prevent the condition, which may

be related to a physiological imbalance of the ratios of the amino acids (Harper et al., '54) although the "essential amino acids" in rations AA<sub>15</sub>, AA<sub>16</sub>, AA<sub>17</sub> and AA<sub>19</sub> were present in relative proportions similar to those found in casein. The concentration of the lipotropic factors, methionine and choline, in these diets was sufficient to prevent fatty livers in mature rats which were not lactating. The development of severely fatty livers during lactation illustrates strikingly that a ration which is satisfactory for the growing or mature animal may be quite inadequate to prevent pathologic changes in tissues when the metabolic economy of the organism is subjected to temporary severe stress.

#### SUMMARY

1. Rats fed protein-free amino acid rations developed hypertrophied and severely fatty livers under the stress of lactation. After lactation the concentration of liver lipids decreased.

2. When lactation of rats was supported by an adequate diet some hypertrophy of the liver was observed but the lipid content of the liver decreased.

#### ACKNOWLEDGMENTS

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# REPRODUCTION OF RATS FED PROTEIN-FREE AMINO ACID RATIONS <sup>1</sup>

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It was previously reported (Schultze, '55) that rats fed protein-free rations containing 16% of a mixture of the 10 "essential amino acids" or 12.2% of a mixture of 16 amino acids could rear their young with a relatively low mortality. The young, however, had subnormal weaning weights and the mothers lost much weight during lactation. In addition, the young born to mothers fed the amino acid rations failed to grow at a normal rate even though suckled by foster mothers fed a natural ration. It appeared that the rations then used were qualitatively or quantitatively inadequate to support satisfactory lactation. Although a few animals of the F<sub>2</sub> generation of rats fed protein-free rations reared their young, there was no assurance that successful reproduction and lactation could be supported by such rations for further generations.

This work was, therefore, extended through the F<sub>4</sub> generation. Moreover, the adequacy for reproduction and lactation of rations containing different levels of 16 amino acids was studied through three generations and comparisons were made with a ration containing 11 amino acids.

## EXPERIMENTAL

*Animals.* The strain of rats and their management were the same as previously reported (Schultze, '55). Some of

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the rats were offspring from mothers fed protein-free amino acid rations, as indicated in the tables, while those identified as P generation were born to mothers fed a ration containing 84% of rolled oats and 6.5% of crude casein and the mixture of lipids, salts, and vitamins described elsewhere (Hallanger and Schultze, '56). These rats were weaned when 21 days old and then fed the amino acid rations while those born to mothers fed the amino acid rations were weaned at 28 days of age. The size of the litters was never reduced. The mothers were housed with males immediately after weaning the first litter.

*Rations.* The composition of the amino acid mixtures I and II,<sup>2</sup> containing 10 and 16 amino acids respectively, has been previously reported (Schultze, '55). Mixture III, containing 11 amino acids, was patterned after mixture II with the following modifications: (1) glycine, DL-alanine and DL-aspartic acid were replaced by equimolar quantities of L-glutamic acid; (2) L-tyrosine was replaced by an equimolar quantity of DL-phenylalanine; (3) L-cystine was replaced by two times its molar quantity of DL-methionine. With these mixtures, the rations were compounded as shown in table 1 of the preceding paper (Hallanger and Schultze, '56). Ration AA<sub>15</sub> contained, per kilogram, 191.2 gm of amino acid mixture III, 718.8 gm of sucrose and the salt, vitamin and lipid components of ration AA<sub>16</sub>.

## RESULTS AND DISCUSSION

*Reproduction and lactation of rats fed 10 amino acids.* Table 1 includes a summary of the observations made with the F<sub>3</sub> (group 1) and the F<sub>4</sub> (group 2) generations of rats fed ration AA<sub>4</sub> containing 15.9% of a mixture of the "essential amino acids" plus 5.2% of diammonium citrate. Of 25 rats, only one failed to conceive; the others delivered 42 litters with a mean of 6.1 young. There was one abortion and two rats died shortly before parturition. The weight gains during

<sup>2</sup> The isoleucine was stated by the manufacturer to contain L-isoleucine, 50%, with D-alloisoleucine, 50%.

TABLE 1  
Reproductive performance of rats fed rations with 10 or 11 amino acids

CATEGORY OF INTEREST	amino acids					
	group 1		group 2		group 3	
Ration; % "essential" amino acids in ration	AA <sub>10</sub> ; 15.94 F <sub>2</sub> <sup>1</sup>		AA <sub>11</sub> ; 15.94 F <sub>2</sub> <sup>1</sup>		AA <sub>11</sub> ; 12.80 F <sub>2</sub> <sup>1</sup>	
Pregnancy	1st	2nd	1st	2nd	1st	2nd
Number of mothers; number of litters born alive	12;12	12;11	13;10	10;9	11;10	8;8
Total number of young born at term	38.4	41.5	54.7	63.2	79.4	71.0
Mortality of young per litter weaned	65	59	79	65	86	63
Mean weight of young, 28 days old, gm	5.3	5.4	7.9	7.1	8.5	7.5
Mean weight loss of mothers, first 7 days, gm	35.4	8.5	0	1.5	10.4	4.8
Mean weight loss of mothers, 28 days, gm	30.8	33.0	20.5	28.1	34.7	37.1
Offspring of P, generation rats previously described (Schultz, '55, p. 571).	8.1	10.0	10.4	13.6	10.0	7.5
Offspring of group 1.	20.6	20.9	13.8	21.5	19.4	16.6
Offspring of group 8 and littermates of groups 9 and 10, table 3.						

TABLE 2  
Reproductive performance of rats fed ration AA<sub>11</sub> containing 12.3% of 16 amino acids

CATEGORY OF INTEREST	amino acids					
	group 4		group 5		group 6	
Ration; % "essential" amino acids in ration	P <sup>1</sup>		F <sub>1</sub> <sup>2</sup>		F <sub>2</sub> <sup>3</sup>	
Pregnancy	1st	2nd	1st	2nd	1st	2nd
Number of mothers; number of litters born alive	12;12	10;9	24;23	19;17	12;12	12;11
Total number of young born at term	64.7	62.1	59.1	71.8	67.5	63.4
Mortality of young per litter weaned	80	64	141	112	84	63
Mean weight of young, 28 days old, gm	6.5	6.9	6.3	7.0	6.6	5.1
Mean weight loss of mothers, first 7 days, gm	28.2	25.0	13.1	8.9	3.6	11.1
Mean weight loss of mothers, 28 days, gm	20.5	32.5	30.2	31.5	25.1	33.4
Offspring of group 5.	29.7	35.8	19.0	18.6	21.8	17.8
Offspring of group 4.			26.1	30.3	25.3	20.5

<sup>1</sup>The rats identified as P generation were born to mothers fed a ration of 84% of rolled oats, and 6.5% of crude casein and the mixture of lipids, salts and vitamins described by Hallanger and Schultze ('56).

<sup>2</sup>Offspring of group 5.

<sup>3</sup>Offspring of group 4.

the pregnancy were below normal particularly in the  $F_3$  generation in which only 5.4 young were born per litter. Except in the first litters of the  $F_3$  generation the mortality of the young was low. Their mean 28-day weight gains were small, particularly among the larger litters (group 2) and the mean total weight of the litters at that time ranged from 162 to 198 gm. Since some of the young, in small litters, reached a 28-day weight of 51 gm, the limited weight gains of most of the suckling rats appear to be due to insufficient amounts of milk secreted by the mother. There was no evidence of gradual deterioration of reproduction and lactation with successive litters or generations.

*Reproduction and lactation of rats fed 11 amino acids.* The total amount of the "essential amino acids" and other sources of nitrogen in the ration are not necessarily the main determinant of the nutritive value of protein-free rations. This is demonstrated by the results obtained with group 3, table 1, which was fed, after weaning, a ration containing 12.8% of a mixture of the "10 essential amino acids" plus 6.3% of L-glutamic acid as a source of additional nitrogen. These animals made greater weight gains during pregnancy, they bore and raised larger litters and the mean total weight (287 gm) of the litters at 28 days of age was 58% greater than that (176 gm) of the litters of mothers in groups 1 and 2 whose ration contained 15.9% of the "essential amino acids." This may be due to more effective utilization of the nitrogen from L-glutamic acid than from diammonium citrate although the total amount of nitrogen contributed by amino acids was similar in both cases, 2.02% in ration AA<sub>4</sub> and 2.19% in ration AA<sub>18</sub>. It is more likely that the relative proportions of the various amino acids in ration AA<sub>18</sub> were more suitable. The molar ratios of the various amino acids to tryptophan (see Flodin, '53) in rations AA<sub>4</sub> and AA<sub>18</sub> are respectively: arginine 1.17 and 2.67; histidine 2.65 and 1.99; isoleucine 7.79 and 11.2; leucine 6.23 and 10.64; methionine 4.11 and 5.79; phenylalanine 5.57 and 7.58; threonine 8.58 and 7.42; valine 12.2 and 13.6; lysine 7.23 and 4.6. Of these, the relative

amounts of methionine and phenylalanine appear to be of minor importance because in ration AA<sub>16</sub> which supported a performance similar to ration AA<sub>18</sub>, the corresponding molar ratios were 3.62 and 4.84 respectively. Since the absolute amounts of histidine, isoleucine, methionine, threonine, phenylalanine, tryptophan, valine and lysine are greater in ration AA<sub>4</sub> than in ration AA<sub>18</sub>, while those of arginine and leucine are 35 and 16% smaller respectively, it appears that a qualitatively "better balance" among the various amino acids is primarily responsible for the better reproduction and lactation supported by the amino acid mixture III.

*Reproduction and lactation of rats fed 12.2% of a mixture of 16 amino acids.* Previous observations with a few animals (Schultze, '55, table 4, group 8) had suggested that rats born to mothers fed 12.2% of a mixture of 16 amino acids incurred a very low mortality. Repetition and extension of this work with larger numbers of animals, covering two litters each from a parental and two successive filial generations confirm the preliminary observations as summarized in table 2. In comparison with groups 1 and 2 (table 1) the weight gain of the mothers during pregnancy was in most instances higher; the weaning weights of the young were essentially the same but the loss of weight of the mothers during lactation was much greater. Most of the weight loss occurred during the first 7 days after parturition at a time when milk production is not at a maximum. The mean weight gains of the second litters were always greater than those of the first litters. There was no evidence in successive pregnancies or generations of a progressive deterioration of the ability to reproduce or lactate.

Although ration AA<sub>15</sub> contained only 7.47% of the "essential amino acids," the reproductive performance and lactation were not inferior to those observed with ration AA<sub>4</sub> which contained 15.9% of the same amino acids. The mean total 28-day weight of the litters from all groups fed ration AA<sub>15</sub> was 194 gm compared to 176 gm observed with ration AA<sub>4</sub>.

This again suggests that the "balance" of the amino acids was better in the mixture II.

*The effect of increasing the amino acid content of the ration.* Since the small weight gains of the young and the large weight losses of the mothers during lactation may have been the result of a suboptimal intake of amino acids, the effect of increasing the amino acid content was investigated with 4 groups of rats. During growth, the first pregnancy and lactation group 7 (table 3) was fed ration AA<sub>15</sub>; during this time the animals represented a replication of the experiments with group 4 (table 2) and the results obtained were very similar. Immediately after weaning of the first litter and throughout the second pregnancy and lactation, the rats of group 7 were fed ration AA<sub>16</sub> which contained 50% more of the amino acid mixture II. During the second pregnancy, these rats made greater weight gains, their young attained about 50% greater weaning weights and the weight losses of the mothers were smaller. This superior reproductive and lactation performance was maintained through two filial generations, namely in groups 8 and 9 (table 3). Although the mean 28-day weight of the young of the F<sub>2</sub> generation was smaller than observed in the F<sub>1</sub> generation (group 8), this cannot be interpreted as a progressive deterioration of the lactation with successive generations because the mean total weight of the litters in both pregnancies of each group was essentially the same, 300 to 329 gm. When the amino acid content of the ration was increased to 24.4% (group 10, table 3) the nutritive value of the ration was not further increased as measured by the weight gains during pregnancy, survival and total mean total weight of the litters at weaning (290 to 299 gm) or the weight change of the mothers during lactation. The number of litters available in these experiments was too small for valid application of more critical methods for evaluation of lactation (Schultze, '54).

*The effect of five non-essential amino acids on reproduction and lactation.* A comparison of the results obtained with group 3 (table 1) and group 9 (table 3) which were comprised

TABLE 3  
Reproductive performance of rats fed rations containing 12.2 to 24.4% of 16 amino acids

CATEGORY OF INTEREST	GROUP 7				GROUP 8		GROUP 9 <sup>1</sup>		GROUP 10 <sup>1</sup>	
	AA <sub>10</sub> ; 12.2		AA <sub>10</sub> ; 18.3		AA <sub>10</sub> ; 18.3		AA <sub>10</sub> ; 18.3		AA <sub>10</sub> ; 24.4	
Ration; % amino acids										
Generation fed amino acid ration										
Pregnancy			P <sup>2</sup>		P <sup>2</sup>		P <sup>2</sup>		P <sup>2</sup>	
Number of mothers; number of litters born alive			1st	2nd	1st	2nd	1st	2nd	1st	2nd
Mean weight gain during pregnancy, gm			11; 11	11; 11	10; 10	10; 10	9; 9	9; 9	10; 10	10; 9
Total number of young born at term			59.5	75.9	74.8	79.4	76.8	73.1	70.8	65.4
Mean number of young per litter weaned			78	80	83	81	80	68	78	66
Mortality of young in 28 days, %			7.0	7.3	7.9	6.9	8.7	7.4	7.3	6.4
Mean weight of young, 28 days old, gm			1.3	7.6	4.8	13.4	2.5	1.5	4.3	1.7
Mean weight change of mothers, 7 days, gm			28.7	43.4	41.7	46.1	36.2	40.6	39.7	46.7
Mean weight change of mothers, 28 days, gm			-20.7	-12.4	-2.2	-6.0	-2.1	-4.3	-3.6	+1.2
			-28.4	-15.0	+4.9	-3.2	+3.1	-5.3	-0.9	+1.4

<sup>1</sup>Animals in groups 9 and 10 were littermates of those of group 3, table 1.

<sup>2</sup>The rats identified as P generation were born to mothers fed a ration of 8.4% of rolled oats and 6.5% of crude casein and the mixture of lipids, salts and vitamin described by Itallinger and Schultz (1956).

<sup>3</sup>Offspring of group 7, second litters.

<sup>4</sup>Offspring of group 8.

<sup>5</sup>Includes third litter of one rat which aborted the second litter.



of littermates permits evaluation of the combined effects of alanine, aspartic acid, tyrosine, glycine and cystine on the nutritive properties of the ration. The rations of both groups were identical with respect to total nitrogen and the amounts and proportions of 8 of the "essential amino acids"; in the ration of group 3, L-glutamic acid was substituted for equimolar quantities of alanine, glycine, and aspartic acid; phenylalanine was similarly substituted for tyrosine and two moles of methionine replaced one mole of cystine in the ration of group 9. In both groups, the mortality of the young was low, there was no significant difference in weight gains during pregnancy or of the suckling young of the first litters which contained about the same mean number of young weaned in both groups. The second litters in group 9, however, reached significantly higher ( $P < 0.01$ ) weights in 28 days. In addition, the mothers in group 9 lost much less weight than those in group 3. The presence of glycine, alanine, aspartic acid, cystine, and tyrosine in the diet appeared to support somewhat better lactation and eased its stress on the mother.

The high percentage of young weaned during several generations fails to support the view (Moruzzi et al., '54; Piccioni et al., '51) that unknown compounds associated with proteins are essential for survival of the young rat.

*The effect of increased lipid content on the ration.* Rose et al. ('54) have shown that young men fed amino acid diets require a high caloric intake for the maintenance of nitrogen equilibrium. Since the rations used in the present and preceding (Schultze, '55) studies contained only 3% of a lipid component, 6 rats from group 5 were fed, immediately after birth of the third litters, ration AA<sub>17</sub> in which 100 gm of hydrogenated vegetable fat<sup>3</sup> replaced 100 gm of sucrose per kilogram of ration AA<sub>15</sub>. All of the 40 young born survived and reached a mean 28-day weight of 31.0 gm compared to 30.2 and 31.5 gm attained by the young of the first two litters. During lactation the mothers lost a mean of 25.8 and 39.3 gm in 7 and 28 days respectively. The addition of fat and the

<sup>3</sup> Crisco.

increased caloric density failed, therefore, to support better lactation or to ease its stress.

*Post-weaning weight gains.* None of the rations supported maximum post-weaning weight gains. When an adequate natural ration is fed to this strain of rats, the females gain about 120 gm, the males about 180 gm in 6 weeks after weaning. The mean weight increments in grams in 6 weeks of the various groups fed amino acid rations were: group 1, 65.0; group 2, 82.6; group 3, 93.4; group 4, 109; group 5, 95.2; group 6, 88.6; group 7, 105; group 8, 95.1; group 9, 104; group 10, 98.2.

Among the rats fed the 12.2% amino acid rations, those of groups 4 and 7 whose mothers received the natural ration made significantly greater weight gains ( $P < 0.02$ ) than those of groups 5 and 6 whose mothers consumed amino acid rations. The best comparison of the growth response can be made with groups 3, 9, and 10 which were comprised of littermates. The rats fed a ration containing 18.3% of a mixture of 16 amino acids (group 9), made 6-week post-weaning weight gains that were significantly greater ( $P < 0.05$ ) than those receiving only 11 amino acids (group 3). The weight gains of group 10 fed 24.4% of a mixture of 16 amino acids were intermediate between those of the other two groups, but not significantly different.

Similar comparisons were also made with groups of 9 to 10 males that were littermates of the females of groups 3, 9, and 10. Those fed ration AA<sub>16</sub> and AA<sub>15</sub> made the same mean 6-week gains, 147 and 146 gm, while those fed ration AA<sub>18</sub> gained only 129 gm, a significantly smaller amount ( $P < 0.01$ ). Although young rats consuming the amino acid rations directly did not consistently increase their daily weight by more than 2.5 gm (females) to 3.5 gm (males), many litters had total daily weight increments of more than 10 gm throughout the period when they subsisted solely on the milk of mothers fed amino acid rations. Under the proper stimulus for food consumption, such as induced by lactation, these rations can, therefore, support far greater synthetic activity in the organism that is apparent from the weight gains of the individual.

*Other observations.* The difference in tables 1 to 3 between the number of mothers and the number of litters born alive in each pregnancy is due to the occurrence of a few resorptions, abortions or maternal deaths shortly before or after parturition or to failures of the animals to conceive. The incidence of such occurrences was highest, 5 out of 47 cases, in groups 1 and 2; in groups 4 to 6, it was reduced to 5/89; in groups 7 to 9, it was 0/60; and in group 10 there was one abortion. Excluding abortions, the number of young born dead was very small in all groups. The mean weights of the young at birth in groups 1 and 2 were 4.8 gm, in the other groups 5.0 gm.

With all of the amino acid rations, the livers of the mothers became greatly enlarged and very fatty during lactation. The quantitative aspects of this observation will be reported separately (Hallanger and Schultze, '56).

#### SUMMARY

1. Protein-free rations containing mixtures of 10, 11 or 16 amino acids were fed to rats during growth, two pregnancies, and lactation, in one instance for as long as 4 successive filial generations without evidence of gradual deterioration of the reproductive or lactation performance.
2. A ration containing 12.2% of mixture of 16 amino acids supported better lactation than a ration containing 15.9% of a mixture of the 10 "essential amino acids."
3. Lactation was better when the level of the mixture of 16 amino acids was increased from 12.2 to 18.3% but a further increase to 24.4% failed to improve reproduction or lactation.
4. A dietary supply of glycine, alanine, aspartic acid, tyrosine and cystine was not essential for reproduction or lactation but when they were fed the loss of weight of lactating mothers was greatly decreased.
5. The nutritive value of a protein-free ration is affected by the relative proportions in which the individual amino acids are present as well as by the absolute amounts.

6. None of the rations used was completely adequate for optimum preweaning or postweaning weight gains of the young or for the prevention of fatty livers during lactation.

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# UTILIZATION OF D-TRYPTOPHAN BY THE CHICK<sup>1</sup>

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Little agreement exists in the literature regarding D-tryptophan utilization by the chick. In 1944 Grau and Almquist reported that chicks could not utilize D-tryptophan. Wilkening et al. ('47) suggested that some of the D isomer was available to the chick and in 1947 Wilkening and Schweigert reported the utilization of D-tryptophan to be from 17 to 40%. Anderson et al. ('50) reported that, when glucose was the carbohydrate in a niacin-deficient diet, D-tryptophan was not utilized. When starch served as the carbohydrate source, however, utilization of the D isomer occurred. It was postulated by these workers that the use of starch permitted a type of intestinal microflora which could invert the D form. Wilkening et al. ('47) and Wilkening and Schweigert ('47) used starch as the source of carbohydrate. Almquist ('52) has suggested, on the basis of the work of Anderson et al. ('50) that the original report (Grau and Almquist, '44) was correct in indicating no utilization of D-tryptophan by the chick inasmuch as conversion by the microflora can scarcely be considered utilization of the isomer by the animal body. West et al. ('52) reported that both isomers of tryptophan were equally well utilized by the chick. However, these investigators supplied such a very small amount of the total tryptophan as the D isomer that much doubt is cast upon the validity of their conclusions.

<sup>1</sup> The data reported in this paper are taken from a thesis submitted to the Graduate College of the University of Illinois by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.

In every instance in which D-tryptophan has been studied in the chick, the DL mixture has been used as the source of the D isomer. In the case of an amino acid whose D isomer is poorly utilized, growth experiments are not sufficiently sensitive to demonstrate differences when the DL mixture is used. This may explain much of the discrepancy which is apparent in the literature.

TABLE 1  
*Composition of basal diet*

CONSTITUENT	AMOUNT
	%
Corn, ground yellow	60.92
Casein, hydrolyzed <sup>1</sup>	18.00
Gelatin (technical grade)	12.24
Corn oil, refined	3.00
Salts <sup>2</sup>	5.34
Choline Cl	.20
DL-Methionine	.30
Vitamins <sup>3</sup>	+
Total	100.00

<sup>1</sup> Hy-case, a salt-free product of Sheffield Chemical Company, Inc., Norwich, New York, devoid of tryptophan.

<sup>2</sup> Glista ('51).

<sup>3</sup> Vitamins (mg/kg diet): Thiamine-HCl 100; nicotinic acid 200; riboflavin 16.0; Ca-pantothenate 20.0; vitamin B<sub>12</sub> 0.02; pyridoxine-HCl 6.0; biotin 0.60; folic acid 4.0; inositol 100; para-aminobenzoic acid 2.0; menadione 5.0; ascorbic acid 250; alpha tocopherol acetate 20.0; vitamin A acetate 10,000 I.U.; vitamin D, 600 I.C.U.

The study reported herein was undertaken in an attempt to clarify further the extent to which D-tryptophan is utilized by the chick.

#### EXPERIMENTAL

The diet used (table 1) contained hydrolyzed casein <sup>2</sup> and gelatin, as the major protein sources, at the same levels as employed by Snyder ('54). The use of ground yellow corn as the carbohydrate source stemmed from the results of repeated trials in which dextrin, cerelose and ground yellow corn

<sup>2</sup> See footnote 1, table 1, for a description of this product.

were used singly and in combination. In every instance the use of corn as the carbohydrate source promoted superior growth. The reason for the superior performance of the corn diet is not evident. Apparently corn adds something to this diet which is otherwise lacking but whether this is an unidentified factor, an improvement in palatability, an improved amino acid balance or some other factor is not clear. This diet contains approximately 0.036% of tryptophan.

Male chicks originating from a mating of New Hampshire males to Columbian females were used in all trials. They were housed in battery brooders equipped with raised wire floors. Feed and water were supplied ad libitum. In experiments 1 to 4 inclusive, tryptophan was added to the basal diet at the expense of corn. These chicks were placed on the experimental diets immediately after hatching. The chicks injected with tryptophan (experiments 5 to 8) were reared on a complete natural-type diet for 14 days, then weighed and randomly allotted to the various treatments by weight. They were then fasted for 12 hours, fed the tryptophan-deficient diet for 72 hours and then subjected to the various treatments to which they had been allotted earlier. The injections were given subcutaneously in the neck region every three hours, with the exception of the 3:00 A.M. injection. Thus the daily allotment of tryptophan was provided by 7 injections. Physiological saline was used as the carrier.

In order to establish that L-tryptophan was not a contaminant of the samples of D-tryptophan<sup>2</sup> used, two different analyses were performed. These were rotation of plane-polarized light and a microbiological assay using *S. faecalis*.

*Oral administration of tryptophan.* The initial experiment was designed to provide information which would serve as a guide for later experiments. Accordingly the basal diet was supplemented with 0.2, 0.4 and 0.6% of D-tryptophan. If D-tryptophan were not used, no growth would be expected; if

<sup>2</sup> The authors are grateful to Monsanto Chemical Company, who, through the courtesy of Dr. Kenneth Maddy, provided most of the tryptophan used in this study.



utilized at approximately 40% of the L isomer, optimum growth would be expected at between 0.4 and 0.6%; if utilized as efficiently as the L isomer the 0.2 level would be expected to give optimum growth. None of these possible results materialized (table 2). Instead, only slight growth resulted at

TABLE 2  
*Growth of chicks fed D- or DL-tryptophan*

EXPERIMENT NO.	GROUP	SUPPLEMENT TO BASAL	AVERAGE WT.	
			7 days	14 days
			gm	gm
1 (5) <sup>1</sup>	1	0.2% D-tryptophan	48	50
	2	0.4% D-tryptophan	54	63
	3	0.6% D-tryptophan	58	73
				16 days
2 (5)	1	None	47	51
	2	0.2% D-tryptophan	51	58
	3	0.4% D-tryptophan	50	62
	4	0.6% D-tryptophan	57	75
	5	0.8% D-tryptophan	64	97
	6	1.2% D-tryptophan	67	105
	7	1.6% D-tryptophan	61	116
	8	0.4% DL-tryptophan	72	166
				14 days
3 (5)	1	None	45	47
	2	1.8% D-tryptophan	71	124
	3	2.1% D-tryptophan	68	131
	4	2.4% D-tryptophan	60	124
	5	2.7% D-tryptophan	65	126
	6	0.4% DL-tryptophan	65	144
4 (15)	1	None		48
	2	2.1% D-tryptophan	.	134
	3	0.4% DL-tryptophan	.	133

<sup>1</sup> Figures within parentheses indicate the number of chicks per experimental group.

the 0.2% level and while the 0.4 and 0.6% levels caused step-wise increases, growth on the 0.6% level was still far below a normal value.

When these points were plotted they fell on a straight line and analysis of variance revealed the regression sum squares

to be highly significant. Least squares calculations and extrapolation revealed that a chick weighing 140 gm at two weeks would require 1.8% of D-tryptophan. Since only three points were available, and since the line was extended beyond the data, the 1.8% value can be regarded with some skepticism.

In the second experiment D-tryptophan was supplied at 0.0, 0.2, 0.4, 0.6, 0.8, 1.2, and 1.6% and a positive control receiving 0.4% DL-tryptophan was included. Once again the graded response noted in the first experiment was evident. The fitted line had a highly significant regression sum squares and extending the line indicated that 2.6% of D-tryptophan would be necessary in order to demonstrate growth equal to that obtained with DL-tryptophan.

In the third experiment D-tryptophan was present at 0.0, 1.8, 2.1, 2.4 and 2.7% of the diet. The results show that maximum growth was obtained when D-tryptophan was provided at 2.1% of the diet. It is evident, however, that the differences between the 4 lots supplemented with D-tryptophan are very small. If only these data were available one might be justified in concluding that 1.8% of the D isomer would promote maximum growth in the chick. When consideration is given to the second experiment with the suggested value of 2.6% of the D isomer for maximum growth, it seems safer to use the 2.1% level as the tentative minimum requirement on this diet. Fitting a straight line to these data revealed a nonsignificant slope and a curve did not reduce the error mean square so that, statistically, it was impossible to establish a difference between any of these values.

The growth on the 2.4 and 2.7% levels of D-tryptophan, while below that of the 2.1%, does not indicate a toxic effect of D-tryptophan. This lack of toxicity of the D isomer at high levels is in agreement with work by Van Pilsum and Berg ('50) and in disagreement with a report by Albanese and Irby ('43).

It is realized that the optimum level of D-tryptophan may in reality be less than the 2.1% level. To establish this point with more certainty would require a more elaborate experiment. It seemed reasonable, however, using a larger number

of chicks, to establish that 2.1% of D-tryptophan would cause chicks to grow as well as those receiving an adequate amount (0.2%) of the L isomer. Therefore in the 4th experiment D-tryptophan was present at 2.1% and L-tryptophan was provided in excess of the 0.15% required, by the use of 0.4% of the DL-mixture. These data leave little doubt that with this diet the D isomer, if provided in sufficient quantity, can be used by the chick for the synthesis of body tissue and this synthesis occurs at a rate comparable to that of chicks fed the L isomer.

*Parenteral administration of tryptophan.* The finding that orally administered D-tryptophan was poorly utilized by the chick, suggested the feasibility of investigating the reason for this inefficiency. Possible reasons for this inefficient utilization are: (1) The body tissue is unable to freely metabolize the D isomer or, more precisely, is unable to efficiently convert the D form to the L form, or (2) the animal lacks the ability to absorb this amino acid efficiently, or (3) the animal body cannot use the D isomer and the high requirement reflects the inefficient conversion of the D isomer by the intestinal microflora.

While the literature contains evidence that D-lysine is not attacked by D-amino acid oxidase (Klein and Handler, '41; Ratner et al., '43; Neuberger and Sanger, '44), such is not the case with D-tryptophan. This suggested that inefficient conversion within the body was not likely to be a major factor. In contrast to this, difficulty in the absorption of the D-isomer has been reported for many amino acids.

Early work had indicated no difference in the absorption of the various isomers (Berg and Baugess, '32; Chase and Lewis, '34; Featherstone and Berg, '42). More recent studies by Gibson and Wiseman ('51) question the validity of this viewpoint. These workers reported the existence of a specific mechanism for the active absorption of the L-amino acids. Use was made of ligatured loops of rat's intestine *in vivo* and 13 amino acids were studied. In addition Wiseman ('51) found by *in vitro* technique, in which isolated rat intestine was used, that the L-isomers of several amino acids were transferred

against a concentration gradient while the corresponding D isomers were not. In 1953 Agar et al. reported similar findings with the L and D isomers of phenylalanine and histidine.

While these investigations suggested the possibility of somewhat similar studies in the present work, the parenteral administration of the D isomer seemed to offer additional advantages. It was felt that if the intestine were by-passed an answer to (1) and the first part of (3) could be obtained as well as some information as to the absorption of the D isomer.

TABLE 3

*Weight and gain of individual chicks receiving 70 mg of L-tryptophan daily by injection every three hours — Experiment 5*

SUPPLEMENT TO BASAL	TIME IN HOURS				
	0	24	48	72	96
	Weight				
	gm	gm	gm	gm	gm
L-tryptophan	175	185	195	201	202 (27) <sup>1</sup>
L-tryptophan	182	202	208	212	230 (48)
L-tryptophan	190	208	217	225	238 (48)
L-tryptophan	188	201	211	216	223 (35)
None	179	180	178	177	179 ( 0)
None	197	197	198	196	194 (—3)
None	173	174	171	170	171 (—2)

<sup>1</sup> Figures within parentheses represent gain or loss in weight from zero time.

Relatively little work has been done with regard to the injection of individual amino acids. Jackson ('27), Alcock ('34) and Yeshoda and Damodaran ('47) reported that injected tryptophan failed to support growth. On the other hand du Vigneaud et al. ('32, '35 '36) found that rats gained weight if given tryptophan by injection. These papers provided little information as to techniques and it was thus necessary to carry out several preliminary experiments before adopting the procedure described earlier.

The initial work was carried out by injecting the L isomer. Typical results with individual chicks are given in table 3. In the preliminary work the level of 70 mg per day was used be-

cause it represents the calculated amount of L-tryptophan required daily by chicks of the size used. The results showed clearly that the subcutaneous injection of L-tryptophan promoted growth in the chick.

Since the ultimate aim of this study was to compare the utilization of the injected L and D isomers it was necessary to

TABLE 4  
*Growth of chicks injected every three hours with D- or L-tryptophan*

EXPERIMENT NO.	GROUP	TREATMENT	TIME IN HOURS				
			0	24	48	72	96
			Average weight				
			gm	gm	gm	gm	gm
6 (10) <sup>1</sup>	1	None	149	146	147	146	
	2	70 mg L-tryptophan/chick/day	148	161	169	176	
	3	70 mg D-tryptophan/chick/day	153	157	162	165	
7 (6)	1	None	170		162		158
	2	35 mg L-tryptophan/chick/day	170		182		192
	3	70 mg L-tryptophan/chick/day	171		189		209
	4	105 mg L-tryptophan/chick/day	172		193		213
	5	140 mg L-tryptophan/chick/day	171		190		210
	6	175 mg L-tryptophan/chick/day	170		188		212
8 (6)	1	140 mg D-tryptophan/chick/day	211	219	219	223	
	2	175 mg D-tryptophan/chick/day	210	216	222	232	
	3	210 mg D-tryptophan/chick/day	207	219	226	236	
	4	245 mg D-tryptophan/chick/day	211	223	234	245	
	5	280 mg D-tryptophan/chick/day	211	221	230	236	
	6	91 mg L-tryptophan/chick/day	210	223	233	246	

<sup>1</sup> Figures within parentheses indicate the number of chicks per experimental group.

establish the requirement for both isomers for optimum growth when administered by the injection technique. First, however, it seemed desirable to establish that the D isomer was utilized by the chick when given parenterally. Accordingly an experiment was performed in which the L and D isomers were provided at 70 mg per day. Table 4 (experiment 6) contains the relevant data. A highly significant growth response resulted from the injection of the D isomer.

The results make it necessary to re-evaluate much of the previously reported work. Contrary to the conclusions drawn by Anderson et al. ('50) and Almquist ('52) the tissues of the chick can, apparently, metabolize and use D-tryptophan for growth.

A further point worthy of note is that this growth resulted on a level of D-tryptophan equivalent to the level used for L-tryptophan. In contrast, when a level of D-tryptophan is fed which is equivalent to the fed L-tryptophan requirement, no growth results. This would suggest that inefficient conversion within the body is not the major cause for the extremely high requirement for D-tryptophan when given *per os*. It also suggests that, while the microflora may be necessary for the utilization of the orally administered D isomer, this is not because of an inability on the part of the tissues to convert the D isomer. In view of these results the very high requirement for orally administered D-tryptophan appears to be due to the fact that this form is absorbed very inefficiently, if at all.

The utilization of parenterally administered D-tryptophan by the chick having been established, it was possible to determine the requirements for the L and D isomers when given by injection. For the determination of the injected L-tryptophan requirement, graded levels of the L isomer were given ranging from 35 mg per day to 175 mg per day. The results of experiment 7 are given in table 4 and figure 1.

A line fitted to the first three arrays had a highly significant slope while the slope of the line fitted to the last two arrays was non-significant. Calculation of the point of intercept revealed an injected L-tryptophan requirement of approximately 91 mg per day. On the basis of the food consumption for the three highest levels it is calculated that the amount of L-tryptophan required per day would be 46 mg. Hence it appears that the procedure used doubles the chick's requirement for this amino acid.

A similar procedure was followed in the 8th experiment in estimating the requirement for D-tryptophan by the injection technique. The levels ranged from 140 to 280 mg of D-tryp-

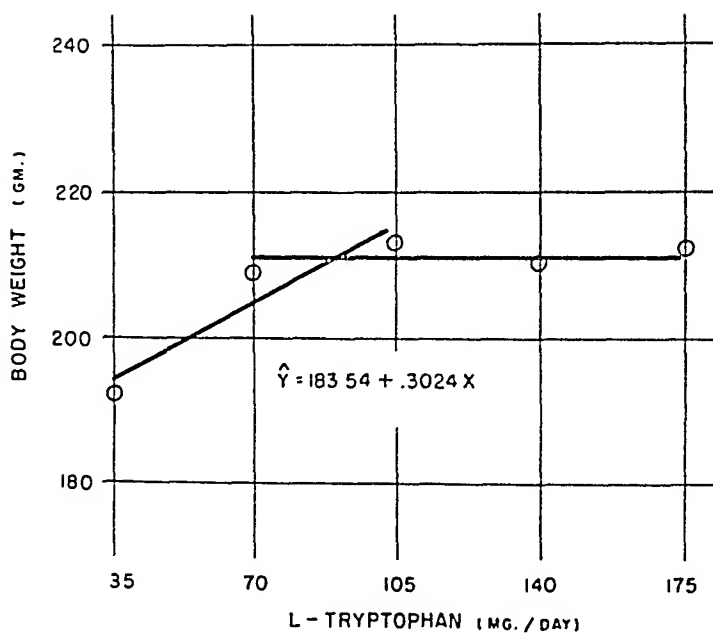


Fig. 1 Requirement for injected L-tryptophan.

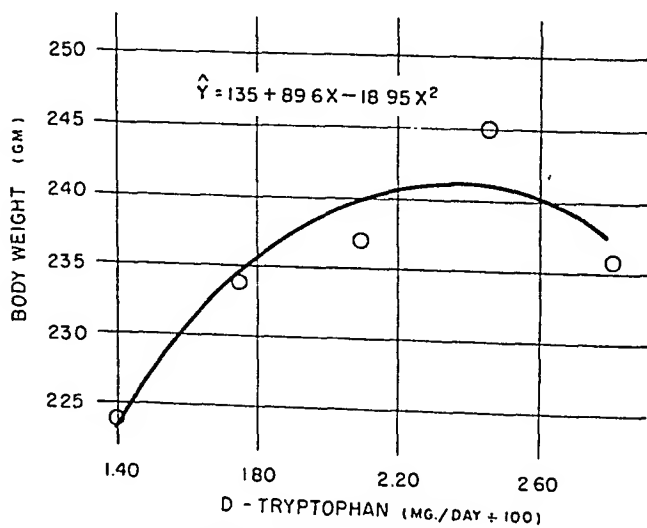


Fig. 2 Requirement for injected D-tryptophan.

tophan per day with increments of 35 mg per day. In addition group 6 was injected with L-tryptophan at the level previously determined as the requirement.

Probably the first point of interest is that growth obtained by the use of the D isomer was equivalent to that noted when the L isomer was used. This demonstrates that the chick's tissues can convert enough D-tryptophan to meet at least 80% of its requirement for this amino acid because, as pointed out earlier, the basal diet contained approximately 0.036% of L-tryptophan which is equivalent to approximately 20% of the chick's requirement for this amino acid.

A quadratic equation (fig. 2) describing the data indicated a requirement for injected D-tryptophan of approximately 236 mg per day. A curve was used in this case because of the decreased growth on the highest level of injected D-tryptophan.

In addition to growth experiments a nitrogen-balance study was conducted and although the results are not presented the data showed clearly that the injection of L- or D-tryptophan caused a highly significant increase in nitrogen retention.

#### DISCUSSION

The data of experiments 2 to 4 indicate that the unsupplemented diet provided tryptophan only slightly in excess of the maintenance requirement of chicks of this size. In order to obtain optimum growth on this diet 0.15% of L-tryptophan must be added (Morrison, '55). To obtain equivalent growth with the D isomer 2.1% is required which is 14 times the L-tryptophan requirement. This points up a weakness inherent in the use of the DL mixture for establishing D isomer utilization. In this case, if one were comparing 0.14% of L-tryptophan to 0.28% of DL-tryptophan, it would be the equivalent of comparing 0.14% of the L isomer to 0.149% of the L isomer. Such a small difference cannot be detected by the usual experimental technique. Moreover, use of the racemic form makes it difficult to estimate the ability of the animal to convert the D isomer beyond 50% of its requirement for that



amino acid. The other 50% is present as the L form since the DL mixture provides essentially equal amounts of the two isomers. Thus expressing the utilization of the D isomer as a percentage is open to question unless the conditions under which the utilization was determined are described.

As a result of this work it can be stated that when the diet contains approximately 0.036% of tryptophan (18% of the L-tryptophan requirement) as the L isomer, the D form will cause optimum growth if provided at 14 times the supplemental L-tryptophan requirement. On this basis the activity of the D isomer is only 7% of that of the L form. This study strongly suggests that in a diet free of the L isomer, the D form could be used as the sole source of this amino acid because, in this diet, it is replacing 0.15% of L-tryptophan. On a diet in which cerelese or dextrin replace corn, the total L-tryptophan requirement is approximately 0.15% (Fisher et al., '55; Griminger, '55). Nevertheless it cannot be stated that the D isomer can meet the entire tryptophan requirement until a diet, devoid of tryptophan and giving satisfactory growth, is used. Work in this laboratory has indicated that in a diet containing dextrin as the carbohydrate source, and thus free of tryptophan, the D isomer does support good growth. Sufficient work has not been done, however, to establish that, on such a diet, the D isomer would support growth equivalent to that promoted by the L isomer. The fact that the D isomer is utilized, even though none of the L isomer is present, is in contrast to work by Celander and Berg ('53a) who reported that, in the mouse, the presence of the L isomers was necessary in order for the D isomers of tryptophan and histidine to be utilized.

These results provide support for those who have previously reported utilization of orally administered D-tryptophan by the chick. The disagreement between these results and the 17 to 40% suggested by Wilkening and Schweigert ('47) may reflect the larger amount of the D isomer being converted in the present study.

As pointed out earlier, the approach taken to this problem precluded associating the entire cause of the inefficient utilization of D-tryptophan with ineffective absorption. It does seem most probable, however, that this step is the primary impediment in the utilization of this D isomer. That it is not due to the lack of ability on the part of the tissues to convert the D isomer to the L form appears evident. This is assuming, of course, that the D isomer is converted to the L form before being built into body tissue, as has been shown for D-histidine by Conrad and Berg ('37) and as suggested by the work of Walser et al. ('50).

With this consideration in mind this study provides an answer to (1) and the first part of (3) listed earlier, namely, that the body tissues are able to utilize D-tryptophan and can do so efficiently.

This is very clearly seen when one compares the requirements of D- and L-tryptophan when fed and when administered parenterally. When these isomers are fed the ratio is approximately 14:1 and when they are injected the ratio is decreased to approximately 2.5:1. On the basis of the food consumed and the *per os* requirement of 2.1%, those chicks receiving the D isomer by injection should have required 420 mg D-tryptophan per day. Instead of this, however, the requirement was approximately 236 mg per day. On the same basis the L-tryptophan requirement should have been 48 mg per day but was shown to be 91 mg per day. Thus the injection technique decreased the chick's total requirement for the D isomer and increased its total requirement for the L isomer. These facts seem to be most readily explainable on the basis of a block in the absorption of the D isomer. This is in agreement with work in the rat by Gibson and Wiseman ('51), Wiseman ('51), Agar et al. ('53).

If absorption is the answer to this problem it is still impossible, on the basis of these data, to say whether the chick cannot absorb the D isomer at all or can absorb it only inefficiently. If the former is true, the growth resulting from high levels of orally administered D-tryptophan must be due

to conversion of the D-isomer to the L form within the intestinal tract, probably by the microflora. If the latter supposition is correct the microflora may or may not play a role in its utilization. Work by Anderson et al. ('50) suggests that the microflora is indeed important in this regard. If the microflora is acting in this way, however, it is only because the D-tryptophan is not reaching the tissues in sufficient quantity and not due to an inability on the part of the tissues to utilize this isomer. The nonessentiality of the microflora for utilization of D-histidine by the rat has been shown by Celander and Berg ('53b).

The greater requirement for injected D-tryptophan over the injected L-tryptophan undoubtedly reflects the loss of the former during conversion. As cited by Krebs ('51) the most active site for D-amino acid oxidase is in the kidney. It is probable that some loss of the keto acid occurs during its transportation from the kidney to the liver.

The injected D-tryptophan gave no evidence of being toxic, except for the highest level, despite the relatively large amounts given. Abderhalden and Tetzner ('35) found that intramuscularly injected D-alanine caused a severe reaction in pigeons. Albanese and Irby ('43) felt their work showed that the D isomers of certain amino acids were toxic. Several others workers have failed to support the conclusions of Albanese and Irby and Van Pilsum and Berg ('50) found the L isomer of methionine to be the more toxic.

Wretling ('52) has pointed out that when the utilization of a D-amino acid is studied, the presence of other amino acids in the D form should be considered. The diet used in this present study contained 0.30% of DL-methionine but how important this was in this instance cannot be stated.

#### SUMMARY

The utilization of orally administered D-tryptophan was studied using growth as the criterion. A diet containing approximately 0.036% of tryptophan was supplemented with

graded levels of the *D* isomer. The results suggest that *D*-tryptophan, if added to the diet of the young chick in sufficient quantity, will promote growth comparable to that obtained by the use of the *L* isomer. The approximate supplemental requirements on this diet for orally administered *L*- and *D*-tryptophan are 0.15 and 2.1% respectively. The utilization of the *D* isomer as found in this study indicates a supplemental requirement for the *DL* mixture of approximately 0.28% of the diet.

Chicks approximately 17 days of age receiving a tryptophan-deficient diet were provided with *L*- or *D*-tryptophan by means of subcutaneous injections. Growth and an increase in nitrogen retention resulted when either isomer was given. The approximate requirements for *L*- and *D*-tryptophan when administered in this way are 91 and 236 mg/chick/day respectively. This work shows that the chick can utilize the *D* isomer without the assistance of the microflora and suggests that inefficient absorption is the cause of the very high requirement for orally administered *D*-tryptophan.

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# FOOD INTAKE AND ESTROGENIC HORMONE EFFECTS ON SERUM AND TISSUE CHOLESTEROL<sup>1,2</sup>

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Cholesterol-fed male rats have shown greater variations in liver cholesterol with different levels of protein intake than have females (Okey and Lyman, '54, '56). In young rats, the sex differences became apparent at the time when males showed the heightened food consumption and growth rate associated with the attainment of puberty. Moreover, they were most marked when the diets contained barely enough good protein to support optimal growth if the total food (and hence calorie) consumption was high. An investigation seemed indicated, to test whether the sex difference was the result of a specific hormone effect or only a corollary of the difference in food intake and growth rate of males and females. Human males are known to have a higher incidence of diseases associated with abnormal tissue cholesterol deposition, whereas human females (as well as female rats) tend to have higher serum cholesterol than males of the same age and diet groups (Gillum et al., '55). It was therefore also considered desirable to find out something about the relationship between circulating and stored cholesterol.

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## EXPERIMENTAL

Two studies were carried out. For the first, 6 groups of 10 150-gm male rats each were studied. Three were placed on the 15% casein-egg albumin diet with 1% cholesterol, previously described<sup>3</sup> (Okey and Lyman, '56), and three on the corresponding 30% protein diet. Two of the groups fed each diet were castrated, and the third served as intact controls. One castrate group on each diet was given 0.05 mg estradiol benzoate in sesame oil, by subcutaneous injection, three times weekly; the other was given sesame oil only. All were fed ad libitum. Weights and food intakes were recorded. Hormone dosage began three days after castration. Three weeks later the rats were autopsied, and the livers and adrenals were prepared for analyses as previously described (Okey and Lyman, '54).

The second study was undertaken because the marked diminution of food intake and growth rate produced by the hormone indicated a need for more data concerning the effect of the quantity of food ingested. For this second study, 110 additional male rats were used. Two-thirds of them were castrated at 150 gm. They were grouped as intact controls, castrate controls, and hormone-dosed castrates. Half of each group was fed the 15% protein diet, half the 30%. Rats of the hormone-dosed castrate subgroup fed 30% protein were used as pacemakers. Two-thirds of the rats from the two intact control groups, the two castrate control groups, and the hormone-dosed castrate group fed 15% protein were given only the mean daily amount of food consumed by the rats of the pacemaker group. In each diet group, the remaining third of the rats was fed ad libitum. The hormone dosage was one-third lower than that given in the first study. After three

<sup>3</sup> The percentage composition of the diets was as follows: (a) "15% protein": egg albumin, 10; vitamin-free casein, 5; fat (Primex), 13.5; sucrose, 64.5; salts, 4; fat soluble vitamin mix, 1; "B" vitamin mix, 1; cholesterol, 1; (b) "30% protein": egg albumin, 25; vitamin-free casein, 5; fat (Primex), 13.5; sucrose, 49.5; salts, 4; fat-soluble vitamin mix, 1; "B" vitamin mix, 1; cholesterol, 1.

weeks' dosage, the rats were sacrificed. Procedures for autopsy and tissue analyses were those previously used, except that blood was collected at decapitation. Serum and tissue phospholipids were determined by the method of Fiske and Subbarow ('25).

### RESULTS AND DISCUSSION

In the two studies, there was little difference between the mean food intakes and growth rates for the corresponding groups of ad libitum-fed rats. Growth data for those of the second study, as shown in figure 1 may therefore be considered typical for all the ad libitum-fed animals.

Gains of control rats were slightly greater with the 15% protein diet than with the 30%. Castrates ate a little less and grew somewhat less well than did intact controls. But

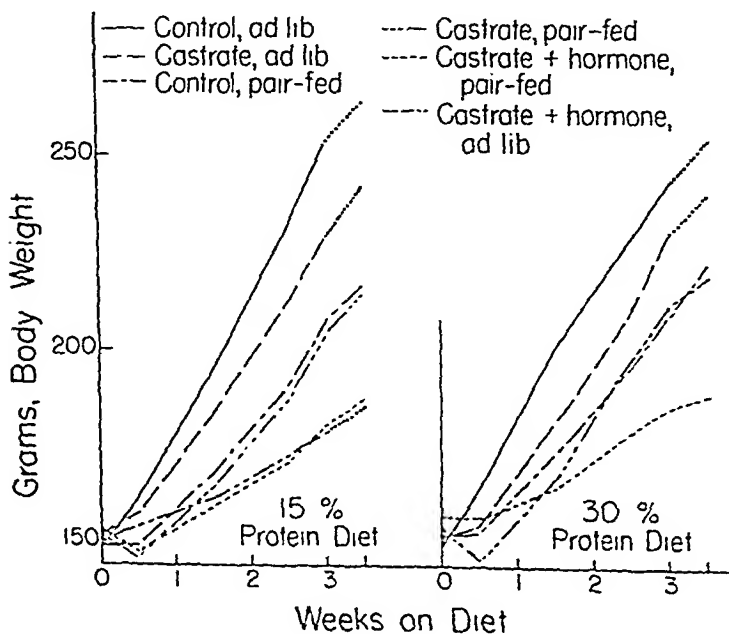


Fig. 1 Mean growth curves for the rat groups of the second study. Lines broken at the ends represent animals fed ad libitum.

the only really significant differences were shown by the hormone-dosed animals. These ate from 75 to 85% as much food as the undosed castrates; but the corresponding weight gains ranged from 33 to 40% and 31 to 45%, respectively, of those of the ad libitum-fed rats on the 15 and 30% protein diets (fig. 1). The decrease in hormone dosage in the second study had no significant effect either on the food intake or on weight gains. Pair-fed intact and castrate rats gained approximately twice as much as their hormone-dosed littermates with the same food intakes.

The *lipid and cholesterol data* for the matched ad libitum and pair-fed rats of study 2 are given in table 1. While the detailed data for the first study are omitted, the fact that the proportionate differences in lipids and cholesterol between the corresponding ad libitum groups in study 1 were much the same as those given in the table deserves mention. This is so because two-thirds of the rats used in study 2 were pair-fed — i.e., each ad libitum subgroup consisted of 6 to 8 rats, while the pair-fed groups were made up of 12 to 14 rats each. The consistency of the findings for the additional subgroups of 10 ad libitum-fed rats each in study 1 therefore assumes some significance. (Actual figures for the two studies were not averaged, both because of the difference in hormone dosage and because, inadvertently, different batches of fat were used in the diets for the two studies.)

In both studies, the intact, ad libitum-fed males given 30% protein had significantly lower liver cholesterol than did those fed 15% protein. Castrates fed the two levels of protein ad libitum showed smaller differences in liver cholesterol than did the corresponding groups of intact males, but slightly greater differences than had been observed in females of the same age and diet groups (Okey and Lyman, '56). The differences in liver lipid and cholesterol, between the two groups of ad libitum-fed, hormone-dosed castrates given 15 and 30% protein, were not significant. The low liver cholesterols in the hormone-dosed rats pair-fed the 15% protein diet may possibly have been associated with their disproportionately

TABLE 1

*Serum and liver lipids*  
(Mean Values)

Liver group	Wt. gm	LIVER					SERUM		
		Total lipid	Cholesterol	Phospho-lipid	Cholesterol to phospholipid ratio		Cholesterol	Phospho-lipid	Cholesterol to phospholipid ratio
		g, moist wt.	g, moist wt.	g, moist wt.	% moist wt.		mg %	mg %	
					15% protein				
<i>Control</i> Ad libitum Pair fed	11.4	15.8	4.21 $\pm$ 0.33 <sup>1</sup>	422 $\pm$ 73	3.55 $\pm$ 0.13	1.14 $\pm$ 0.09	98 $\pm$ 12	146 $\pm$ 16	0.67 $\pm$ 0.13
	9.8	13.2	2.76 $\pm$ 0.15	272 $\pm$ 21	3.74 $\pm$ 0.22	0.76 $\pm$ 0.25	100 $\pm$ 12	129 $\pm$ 10	0.73 $\pm$ 0.06
<i>Castrate</i> Ad libitum Pair-fed	11.0	13.4	3.33 $\pm$ 0.34	356 $\pm$ 34	3.38 $\pm$ 0.18	0.98 $\pm$ 0.10	103 $\pm$ 12	142 $\pm$ 14	0.71 $\pm$ 0.04
	8.8	11.0	2.63 $\pm$ 0.37	233 $\pm$ 30	3.69 $\pm$ 0.13	0.71 $\pm$ 0.10	117 $\pm$ 8	132 $\pm$ 5	0.89 $\pm$ 0.15
<i>Castrate + hormone</i> Ad libitum Pair fed	7.9	11.5	4.29 $\pm$ 0.31	350 $\pm$ 31	3.43 $\pm$ 0.24	1.29 $\pm$ 0.11	284 $\pm$ 30	234 $\pm$ 15	1.22 $\pm$ 0.11
	8.2	9.5	3.20 $\pm$ 0.31	260 $\pm$ 30	3.43 $\pm$ 0.12	0.87 $\pm$ 0.10	352 $\pm$ 37	235 $\pm$ 8	1.49 $\pm$ 0.14
					30% protein				
<i>Control</i> Ad libitum Pair-fed	10.3	10.5	2.97 $\pm$ 0.40	296 $\pm$ 33	3.96 $\pm$ 0.19	0.74 $\pm$ 0.09	102 $\pm$ 9	144 $\pm$ 17	0.72 $\pm$ 0.03
	8.9	9.7	2.62 $\pm$ 0.21	223 $\pm$ 24	4.21 $\pm$ 0.22	0.64 $\pm$ 0.05	138 $\pm$ 13	154 $\pm$ 10	0.89 $\pm$ 0.07
<i>Castrate</i> Ad libitum Pair fed	9.3	10.3	2.98 $\pm$ 0.47	270 $\pm$ 38	3.38 $\pm$ 0.18	0.77 $\pm$ 0.11	120 $\pm$ 10	166 $\pm$ 7	0.77 $\pm$ 0.03
	8.8	10.3	2.67 $\pm$ 0.33	228 $\pm$ 22	4.09 $\pm$ 0.12	0.65 $\pm$ 0.07	163 $\pm$ 14	148 $\pm$ 8	1.09 $\pm$ 0.09
<i>Castrate + hormone</i> Pacemakers	8.8	10.6	4.12 $\pm$ 0.21	362 $\pm$ 20	3.36 $\pm$ 0.11	1.24 $\pm$ 0.07	275 $\pm$ 22	235 $\pm$ 11	1.17 $\pm$ 0.08

<sup>1</sup> Standard error  $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$ .

large initial weight loss after castration. However, older female rats have been observed in this laboratory to show some tendency toward increases in liver cholesterol on long continued feeding of high protein diets.

Most interesting, however, was the contrast between the serum and liver lipid values for the ad libitum-fed and the restricted rats of the corresponding groups. Differences in liver cholesterol with differences in the protein content of the diet were almost eliminated in the rats with restricted food intake. But *serum* cholesterols were, in each case, higher for the restricted than for the ad libitum-fed rats. Values for castrates were approximately as much higher than serum cholesterols for controls as values for females have been observed to be higher than those for males. Rats fed 30% protein showed a greater increase in serum cholesterol with restriction of food intake than did those fed 15% protein.

On the assumption that mobility of circulating cholesterol may possibly be related to phospholipid content of serum, the latter was determined. Serum phospholipids were found to be decreased slightly by restriction of food intake. This meant that, for the 30% protein groups, differences in cholesterol-phospholipid ratios produced by dietary restriction were statistically significant ( $p < 0.05$  and  $p < 0.02$  for the controls and castrates, respectively) in spite of the fact that, as noted earlier, the comparison was made on the basis of data for the comparatively small groups fed ad libitum in the second study.

Concentration of *liver phospholipid* was higher in the *undosed* rats receiving 30% protein than in those receiving 15%. Restriction of food intake seemed to increase the concentration slightly in the intact control and in the castrate rats fed both diets. The net result was a lowering of the cholesterol/phospholipid ratios in the livers of the undosed, restricted rats. The extent of this lowering was slightly greater in the rats fed 15% protein, where liver cholesterols were high in the ad libitum groups. In all of the *hormone-dosed* rats, however, liver phospholipid concentrations were low and serum phos-

pholipid increases were not so great as the increases in serum cholesterol. The cholesterol-phospholipid ratios with estradiol benzoate dosage were, therefore, high and much alike in both serum and liver.

It seems logical, therefore, to consider the hypothesis that the effect of the hormones and that of dietary restriction may be independent, but that, under some conditions, they may augment each other.

In the intact, pair-fed male *controls*, presumably, the growth promoting and testicular hormones were active. The lowering of liver cholesterol, with its concomitant increase in serum cholesterol, could therefore be attributed to the decreased food intake, possibly augmented by some increase in the efficiency of cholesterol absorption.

In the *castrates*, testicular hormones were absent, but adrenal and pituitary hormones were functioning presumably uninhibited by the sex hormones. Nevertheless, the *ad libitum*-fed castrates had liver cholesterols which were about as much lower than those of the intact controls as were their total food intakes, a finding which did not indicate any specific effect of the lack of testicular hormone on liver cholesterol storage.

The increases in *serum cholesterol* with dietary restriction were greater in castrates than in intact animals, a result which might be explained by the absence of a male-hormone effect in lowering circulating cholesterol. The increases in serum cholesterol in the estrogen-dosed castrates were, however, far greater than those in the undosed castrates with the same food intake (75 to 120%). At the same time, *liver* cholesterols in the hormone-dosed rats remained higher than the food intakes and growth rates of these animals might lead one to expect. The hormone seemed therefore to have a specific effect. It was not, however, clear whether it acted by mobilization of dietary cholesterol, presumably for hormone production; by stimulation of cholesterol synthesis; or by producing a decrease in turnover rate which overbalanced the effect of decreased food intake on liver cholesterol accumulation.

As one means of testing for evidence of increased mobilization for hormone secretion, *adrenal cholesterols* were determined (table 2). Adrenal cholesterol concentrations were considerably increased by dietary restriction. This change reflected differences of only 0.2 to 0.5 mg cholesterol per

TABLE 2  
*Adrenal cholesterol*  
(Means and standard errors)

GROUP AND DIET	NO. RATS	WT. OF ADRENALS	CHOLESTEROL	
		mg	% moist wt.	mg
15% protein				
<i>Control</i>				
Ad libitum	6	37.6	3.48 ± 0.36 <sup>1</sup>	1.33 ± 0.19
Pair-fed	12	33.3	4.75 ± 0.36	1.59 ± 0.13
<i>Castrate</i>				
Ad libitum	7	38.5	3.51 ± 0.37	1.39 ± 0.22
Pair-fed	13	36.5	5.21 ± 0.29	1.91 ± 0.12
<i>Castrate + hormone</i>				
Ad libitum	7	50.8	4.79 ± 0.26	2.41 ± 0.11
Pair-fed	12	44.6	5.19 ± 0.27	2.32 ± 0.13
50% protein				
<i>Control</i>				
Ad libitum	6	39.9	3.81 ± 0.51	1.51 ± 0.17
Pair-fed	12	34.2	4.64 ± 0.31	1.59 ± 0.11
<i>Castrate</i>				
Ad libitum	7	42.1	3.31 ± 0.26	1.43 ± 0.21
Pair-fed	14	37.9	4.46 ± 0.32	1.68 ± 0.12
<i>Castrate + hormone</i>				
Pacemakers	13	49.5	4.34 ± 0.14	2.13 ± 0.14

<sup>1</sup> Standard error  $\sqrt{\frac{\sum d^2}{n(n-1)}}$ .

adrenal because of the slightly smaller size of the adrenals in the restricted rats. However, the size of the adrenals was significantly greater (8 to 14 mg) in the hormone-dosed animals, as were the percentage and amounts of cholesterol. Whether or not this indicates that administration of estrogenic hormone stimulated production of cortical hormones from cholesterol, the differences were somewhat larger than

those usually found between adrenals of male and female rats.

The data as a whole indicate that both restriction of food intake and dosage with estrogenic hormone are capable of altering cholesterol transportation and storage. In both cases the primary effect seems to be an increase in the circulating cholesterol. With restriction of total food intake, this increase is greater when the diet is high in protein. With estrogenic hormone, serum cholesterols were so high that relation to diet became doubtful.

That the increase in circulating cholesterol can take place at the same time that liver cholesterol stores are depleted adds one more reason for doubting the value of serum cholesterol determinations as an index of tissue cholesterol retention. The fact that serum cholesterols were consistently increased by restriction of food intake, which meant restriction of cholesterol intake as well, also raises some questions of interpretation of data.

McMillan et al. ('54) have reported increases in serum cholesterol with lowering of food intake in rabbits dosed with set amounts of cholesterol. They found no increase in the incidence or severity of atherosclerosis in their underfed animals. Rabbits are rated as more likely to show tissue cholesterol deposits in response to cholesterol feeding than are human beings, but rats are especially resistant to development of cholesterol lesions. It should be interesting to follow, at frequent intervals, the circulating cholesterol level in human atherosclerosis patients who are consuming high protein diets as a means of controlling weight. Also, obviously, correlation of autopsy data with dietary histories is indicated.

#### SUMMARY

An ad libitum and a pair-feeding study are reported for castrated male rats fed 1% cholesterol with 15 and with 30% protein. In each case one castrate group was treated with estradiol benzoate.



Food intakes of the hormone-dosed castrates were reduced one third to one fourth as compared with ad libitum controls, intact and castrate, respectively. However, when undosed castrates were pair-fed to those dosed with hormone, they gained approximately twice as much.

The ad libitum intact controls showed the usual higher liver cholesterol with the 15 than with the 30% protein diet. Variation in liver cholesterol with protein content of the diet was less in castrates than in controls.

In the undosed rats restricted as to food intake, *serum cholesterols* were higher and *liver cholesterols* lower than in the ad libitum-fed rats. The increase in serum cholesterol with restriction of food intake was greater in the rats fed 30% protein.

*Serum cholesterols* were very high and adrenal cholesterols were increased in the hormone-dosed rats, but *liver cholesterols* for both diet groups were within the ranges of those of the intact controls fed 15% protein. Data are discussed in terms of relationship of food and cholesterol intake to serum and liver cholesterol.

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# THE RELATION OF DIETARY SUPPLEMENTS AND TISSUE METABOLITES TO GLYCINE TOXICITY IN THE CHICK<sup>1</sup>

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## INTRODUCTION

Glycine toxicity in the chick can be prevented by the administration of high levels of folacin (Naber, Snell and Cravens, '52; Machlin, Lankenau, Denton and Bird, '52), but it is not known why glycine is toxic nor how folacin enables the chick to metabolize excess glycine successfully. Folacin is directly or indirectly concerned with several metabolic reactions of glycine, including conversion to serine (Elwyn and Sprinson, '50; Broquist, '49), the synthesis of purine bases and hence of uric acid (Shemin and Rittenberg, '47; Buchanan, Sonne and Delluva, '48) and the incorporation of formate into serine and heme (Plaut, Bethel and Lardy, '50). Pathways not thought to be mediated by folacin involve the conversion of glycine to creatine (Bloch and Schoenheimer, '41), glyoxylic acid, or oxalic acid (Ratner, Nocito and Green, '44). Folacin-deficient chicks might therefore have a diminished capacity for the metabolism of glycine through normal channels, and hence toxicity could result either from the presence in the body of excess glycine itself, or of a metabolite arising from reactions

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that take precedence when folacin is limiting. In the present study measurements have been made of likely metabolites in the tissues of chicks fed excess glycine, and attempts made to alter the severity of the toxicity with appropriate nutrients.

#### EXPERIMENTAL

Day-old New Hampshire  $\times$  Single Comb White Leghorn crossbred chicks of both sexes obtained from dams fed ration B-1 of Robblee, Nichol, Cravens, Elvehjem and Halpin ('48) were reared in electrically heated batteries with raised screen floors. Feed and water were supplied ad libitum. Ten to 25 chicks were allotted at random to each of the experimental groups. The composition of the basal ration was sucrose 600, hot-alcohol extracted casein 180, gelatin 100, DL-methionine 3, soybean oil 50, fish oil (1500-A, 300-D) 5, salts V (Briggs, Luckey, Elvehjem and Hart, '43) 60 and choline chloride 2. Vitamins were added as follows in milligrams per kilogram of ration: thiamine  $\cdot$  HCl 6.0, riboflavin 6.0, niacin 100.00, calcium pantothenate 20.0, pyridoxine  $\cdot$  Cl 4.0, biotin 0.2, 2-methyl-1,4-naphthoquinone 0.5, alpha tocopherol acetate 3.0, para amino-benzoic acid 100.0 and meso inositol 1000.0. Vitamin B<sub>12</sub> was supplied in crude form to provide 50  $\mu$ g/kg of ration. This ration did not contain any added folacin. All supplements were included at the expense of sucrose.

Blood glucose determinations were made with the anthrone reagent as described by Durham, Bloom, Lewis and Mandel ('50). Liver glycogen analyses were made by the direct method of Seifter, Dayton, Novic and Muntwyler ('50). Uric acid was measured according to the method of Brown ('45). Blood glycine levels were measured by the method of Alexander, Landwehr and Seligman ('45). Para-hydroxyphenyl compounds were determined in blood serum as described by Seydl, Thiele and Boguth ('50) with para-hydroxyphenyl pyruvic acid as a standard. This latter was synthesized and purified according to the procedure described by Herbst and Shemin for phenylpyruvic acid ('43). Glyoxylic acid was prepared by the method of Benedict ('09).

Serum calcium determinations were made by the Clark-Collip modification of the Kramer-Tisdall method published by Hawk, Oser and Summerson ('49). Microbiological assays for folacin were made on the liver, kidney, pancreas and spleen of chicks as described by Sunde, Cravens, Bruins, Elvehjem and Halpin ('50), except that spleen and pancreas samples were autolysed at pH 7.0 to secure maximum liberation of bound folacin. In one experiment the toxicity of certain compounds was tested by depositing aqueous solutions directly into the crop of the chick with the aid of a syringe fitted with a small plastic tube.

#### RESULTS

As in previous studies the addition of 5% of glycine to a semisynthetic low-folacin diet depressed growth and produced symptoms of toxicity such as nervousness, tremors, protruding eyes, and occasional paralysis, whereas in the presence of folacin there were no symptoms nor was growth affected by glycine (table 1). The most significant biochemical changes observed were in liver glycogen, which was depressed by glycine and increased by folacin. Blood glucose, uric acid, calcium, and *p*-hydroxyphenyl compounds were unaffected by either glycine or folacin. Blood glycine was increased about three-fold when glycine was fed, but the addition of folacin to the diet did not reduce these levels (table 1). Conversely, the addition of a toxic level of glycine to a crude practical ration failed to depress the folacin content of the tissues, and in the liver and pancreas, folacin was actually somewhat higher in the birds fed glycine (table 2).

The specificity of glycine toxicity was demonstrated by the failure of serine to depress growth or to cause any symptoms of toxicity (table 3; also Naber et al., '52) while the growth-depressing effects of alanine and tyrosine were not corrected by folacin (table 3). Furthermore, the birds fed these latter acids did not exhibit typical symptoms of glycine toxicity.

In other trials a dietary supplement of the creatine precursors arginine or methionine or both, largely prevented

TABLE 1

*The effect of folacin and glycine on blood and organ levels of various components*

GROUP NO.	SUPPLEMENT TO BASAL RATION		AV. WEIGHT AT 2 WKS. (ALT. EXPS.)	AVERAGE VALUES <sup>1</sup> AFTER 2 WEEKS										Serum calcium Exp. C-16
	Folacin	Glycine		Blood Exp. C-7	Glucose Exp. C-8	Liver glycogen Exp. C-15	Blood uric acid Exp. C-9	Blood glycine Exp. C-12	Blood p. hydroxyphenyl compounds		Serum calcium Exp. C-11			
									mg %	mg %		mg %	mg %	
1	..	..	82	200	241	2.7 <sup>2</sup>	8.3	11.1 <sup>2</sup>	14	15	10.2			
2	..	5	62 (Severe toxicity symptoms)	206	210	1.3	8.6	28.1	14	16	10.4			
3	10	..	109	237	208	5.6	8.1	11.9	18	17	10.4			
4	10	5	110	222	201	3.2	9.2	33.9	14	16	10.3			

<sup>1</sup> Each value represents the average of 4 to 10 determinations.

<sup>2</sup> Analysis of variance indicates that statistically significant differences at the 1% level exist, with liver glycogen, between group 1 and groups 2 and 3; with blood glycine, between group 1 and groups 2 and 4.

the toxicity symptoms of glycine, but did not restore the growth of the glycine-fed chicks.

Two per cent of sodium formate caused a small increase in growth when added to the folacin-deficient basal ration in each of three trials (table 4), but showed no beneficial effect

TABLE 2.

*Folacin content of organs from chicks fed glycine*

SUPPLEMENT TO PRACTICAL TYPE RATION (Exp. C-5)	AV. WEIGHT (AFTER 10 DAYS)	AVERAGE FOLACIN CONTENT <sup>1</sup> ( $\mu$ G/GM FRESH TISSUE)			
		Liver	Kidney	Pancreas	Spleen
	<i>gm</i>				
None	357	7.1 <sup>2</sup>	1.9	0.23 <sup>2</sup>	0.12
10% Glycine	302	12.3 <sup>2</sup>	1.9	0.39 <sup>2</sup>	0.09

<sup>1</sup> Each value represents the average of 15 determinations.

<sup>2</sup> Analysis of variance indicates differences statistically significant at 1% level.

TABLE 3

*Effect of glycine, DL-serine, DL-alanine and L-tyrosine on chick growth*

SUPPLEMENT TO BASAL RATION		AV. WEIGHT AT 2 WEEKS			GLYCINE TOXICITY SYMPTOMS
Folacin	Amino acid	Trial C-8	Trial C-12	Trial C-11	
<i>mg/kg</i>		<i>gm</i>	<i>gm</i>	<i>gm</i>	
	None	96	81	75	..
	5.0% Glycine	69	57	55	Yes
	5.0% DL-Serine	92			No
	5.0% DL-Alanine		59		No <sup>1</sup>
	5.0% L-Tyrosine			56	No
10.0	None	127	93	98	
10.0	5.0% Glycine	120	101	91	No
10.0	5.0% DL-Serine	126			No
10.0	5.0% DL-Alanine		70		No <sup>1</sup>
10.0	5.0% L-Tyrosine			64	No

<sup>1</sup> Slow painful gait.

in the presence of added folacin. Larger quantities of sodium formate were found to be toxic and did not respond to folacin supplementation. Furthermore, dietary formate failed to influence the toxic effect of glycine.

Figure 1 suggests a relationship between dietary riboflavin and glycine toxicity. While growth on the glycine-containing

ration was poor at all riboflavin levels, symptoms of glycine toxicity were less severe at the lower levels of riboflavin.

The effects of single doses of glycine, glyoxylic acid and oxalic acid on chicks are shown in table 5. Glyoxylic acid and

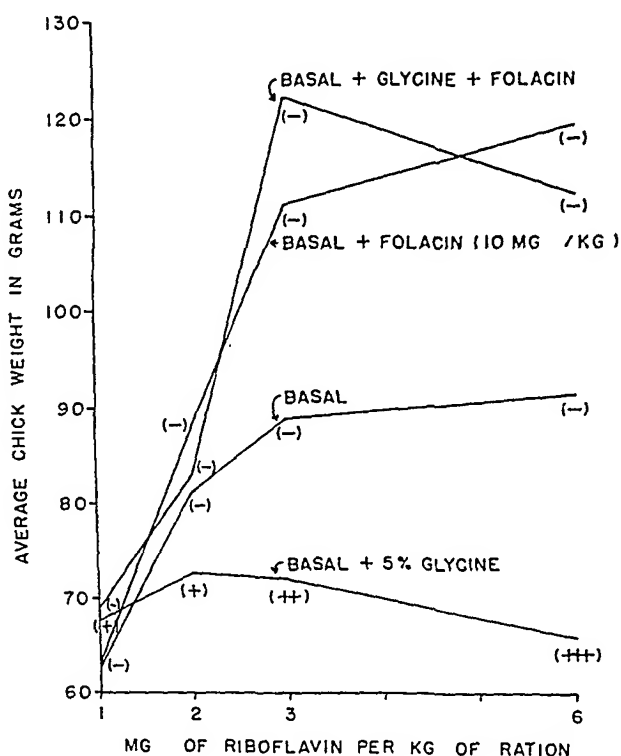


Fig. 1 Effect of dietary riboflavin level on glycine toxicity (exp. C-16). Minus sign (—) indicates absence of toxicity, plus sign (+) indicates presence of toxicity symptoms and severity of symptoms is indicated by increased number of plus signs (++) or (+++).

oxalic acid proved to be toxic in smaller amounts than glycine, but with symptoms different from those produced by glycine. Dietary folacin failed to protect the chicks against glyoxylic or oxalic acid, but as usual helped the chicks withstand an otherwise lethal dose of glycine.

The feeding of ascorbic acid, niacin, riboflavin, pyridoxine, vitamin B<sub>12</sub>, and alpha tocopherol in addition to the quantities of these vitamins contained in the basal ration had no effect on glycine toxicity where folacin-deficient rations were employed.

TABLE 4

*Effect of sodium formate on chick growth and on glycine toxicity*

GROUP NO.	SUPPLEMENT TO BASAL RATION			AV. WEIGHT AT 2 WEEKS			GLYCINE TOXICITY SYMPTOMS
	Folacin	Glycine	Na formate	Trial C-14	Trial C-15	Trial C-16	
	mg/kg	%	%	gm	gm	gm	
1				83 <sup>1</sup>	95 <sup>1</sup>	79 <sup>1</sup>	..
2		5.0		59	71	..	Yes
3			2.0	93	104	94	..
4		5.0	2.0	..	67	..	Yes
5			4.0	80	..	..	..
6	10.0			120	125	127	..
7	10.0	5.0		105	112	..	..
8	10.0		2.0	106	115	119	..
9	10.0	5.0	2.0		107	..	..
10	10.0		4.0	85	..	..	..

<sup>1</sup> Analysis of variance, combining three trials, indicates differences statistically significant at 1% level between group 1 and groups 3, 6 and 8.

TABLE 5

*Effect of dietary folacin on the toxicity of glycine, glyoxylic acid and oxalic acid given in a single oral dose*

(Exp. C-17)

DOSE AS % OF BODY WEIGHT	BASAL		BASAL + 10 MG FOLACIN/KG	
	% Survivors (48 hours after treatment)	Av. gain or loss in weight of survivors	% Survivors (48 hours after treatment)	Av. gain or loss in weight of survivors
		gm		gm
None	100	+ 14	100	+ 21
Water	100	+ 14	100	+ 21
0.5% Glycine	100	+ 3	100	+ 12
1.0% Glycine	0		80	+ 5
0.1% Glyoxylic acid	60	— 5	60	— 5
0.2% Glyoxylic acid	0		0	
0.1% Oxalic acid	0		0	
0.2% Oxalic acid	0		0	



## DISCUSSION

The present experiments do not settle the question as to why glycine is toxic, nor do they reveal exactly how folacin counteracts this toxicity. They do, however, restrict the possibilities somewhat. The toxicity does not appear to be due to high levels of blood glycine *per se*, since the levels of blood glycine were as high in normal chicks fed glycine and folacin as in abnormal birds fed glycine supplements alone. Nor was the effect one of inducing a folacin deficiency, since tissue folacin was at least as high in the glycine-fed birds as in the controls. The specificity of glycine toxicity is apparent from the fact that the symptoms were not duplicated by alanine or tyrosine nor by the metabolites serine, formate, oxalic acid or glyoxylic acid. Most of these latter substances depressed growth, but depression was not corrected by folacin.

The non-toxicity of serine is particularly suggestive. Serine contributed as much excess metabolizable nitrogen as glycine and hence the adverse effect of the latter can hardly be ascribed to an overloading of the mechanism for nitrogen elimination (uric acid production). Furthermore, the ready conversion of glycine to serine (Elwyn and Sprinson, '50), a reaction that requires folacin, raises the possibility that this might be a major means by which excess glycine is metabolized. Totter, Kelley, Day and Edwards ('50) incubated radioactive glycine with liver homogenates from folacin-deficient chicks and recovered 42% of the activity in isolated glycine and 23% in serine. When normal chick liver homogenates were employed, only 15% of the activity was found in glycine, but 36% was found in the isolated serine. The high levels of folacin required to counteract glycine toxicity, and the present finding that folacin levels in liver usually increase with glycine feeding all suggest that the excessive quantities of glycine might be removed by conversion to serine. However, in the present experiments the extent of this type of removal, as pointed out previously, was not sufficient to decrease the levels of blood glycine, and the symptoms of toxicity would therefore seem best explained as the result of alternate meta-

bolic products that accumulate when the conversion of glycine to serine is impaired. A glycine oxidase which requires flavin-adenine dinucleotide has been found in mammalian tissues (Ratner, Nocito and Green, '44), the immediate product being glyoxylic acid, which can go to oxalic acid by an aldehyde oxidase. If these systems were of significance in the chick, they might help explain why symptoms of glycine toxicity were more severe when dietary riboflavin was increased, even though the specific symptoms were not duplicated when toxic levels of glyoxylic and oxalic acids were given orally. It is of interest that variations in the other vitamins tested did not modify the toxicity of glycine.

#### SUMMARY

1. Although symptoms of glycine toxicity in chicks were prevented by folacin, dietary glycine failed to depress the folacin content of the tissues, nor did folacin depress the level of glycine in the blood. Liver glycogen was depressed by glycine and elevated by folacin; blood glucose, uric acid, calcium and *p*-hydroxyphenyl compounds were unaffected by either.

2. Unsuccessful attempts to simulate glycine toxicity with possible metabolites included the administration of oxalic acid and glyoxylic acid, while the simultaneous feeding of glycine and formate failed to alter the course of glycine toxicity. The feeding of methionine and arginine minimized the symptoms but failed to restore growth.

3. Serine was innocuous at levels equivalent to those that produced glycine toxicity. Tyrosine and alanine depressed growth without correction by folacin. Preliminary evidence suggested that high levels of riboflavin might aggravate the the symptoms of glycine toxicity.

#### ACKNOWLEDGMENTS

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# MUSCULAR DEGENERATION IN CHICKENS FED DIETS LOW IN VITAMIN E AND SULFUR<sup>1,2</sup>

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During a study on sulfate metabolism (Machlin, '55), white striations were observed in the muscles of the breast and the legs of young chickens fed diets low in sulfur. The lesions resembled those reported by Dam et al., ('52). In the studies presented here the protective effect of cystine and vitamin E reported by these Danish workers was confirmed. In addition the effect of methionine and the antioxidant diphenyl-p-phenylenediamine (DPPD) in the prevention of the muscular degeneration and the negative or slight influence of variety of other compounds are reported.

## EXPERIMENTAL

In each experiment, day-old, New Hampshire X Silver Cornish crossbred female chicks were fed the basal diet for one week. They were then weighed, divided into groups of approximately equal weight distribution, and fed the experimental diets for three weeks. The birds were reared under electrically heated brooders in wire-floor cages in an air-conditioned room. At the end of the experiment the birds were killed and the muscles examined and scored for severity of muscular degeneration. Tissues for histological examination

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were dissected immediately after death and placed in 8% formalin.

The basal diet which contains 15% casein and 10% gelatin as the protein sources, is essentially the same as that used by Gordon and Sizer ('55), with 2.62 gm  $MgCO_3$ , 0.37 gm  $MnCl_2$  and 0.045 gm copper acetate replacing the corresponding sulfate salts.

This diet contains about 0.54% of methionine, 0.06% of cystine and no appreciable sulfate S. This is less than the recommended level of 0.8% of the sulfur-containing amino acids considered necessary for optimum growth of the young chicken (Almquist, '52). All diets were stored at 2° C. and mixed freshly for each experiment.

#### RESULTS

*Experiments 1, 2 and 3.* The original purpose of experiments 1 and 2 was to determine whether the addition of sulfate S to a low-sulfur diet would stimulate the early growth of the chick. These data are reported elsewhere (Machlin, '55). In these experiments the basal diet contained 4% of cottonseed oil and 10 mg/kg of alpha tocopheryl acetate.

At 4 weeks of age most of the birds fed the basal diet had white parallel striations in the breast muscle in the direction of the muscle fibers. Occasionally these lesions were seen in the leg muscles. Other skeletal muscles were affected only rarely. When examined histologically, it was observed that there were wide linear areas of degeneration involving several adjacent muscle fasciculi which accounted for the white or light colored bands noted grossly. The degeneration was a waxy or hyaline type characterized by fragmentation, hyalinization, loss of striation, multiplication of sarcolemmal cells, infiltration by heterophil cells and clumping of fibers into eosinophilic masses. There was some calcification in specimens with a more severe reaction. Both degeneration and regeneration were apparent in single tissues. For instance, in a part of one specimen there was no cellular reaction in some areas but very diffuse atrophy and disappearance of the muscle fibers and cells. In other

parts of this specimen there was active regeneration with proliferating muscle nuclei.

No significant changes were observed in specimens of gizzard or heart muscle or of liver, kidney, pancreas or spleen tissues from chickens with severe muscular degeneration. No evidence of encephalomalacia, edema or exudative diathesis was seen. Moreover, the birds showed no signs of weakness of the leg or breast muscles. Mortality was low in all experiments.

TABLE 1

*Incidence of muscular degeneration in 4-week-old chicks fed a diet containing cottonseed oil*

SUPPLEMENT	EXPERIMENT		
	1 <sup>1</sup>	2 <sup>1</sup>	3 <sup>2</sup>
None	23/25 <sup>2</sup>	12/17	9/10
Na <sub>2</sub> SO <sub>4</sub> , 0.5%	10/11	6/17	6/10
DL-Methionine, 0.3%	1/15	0/16	1/10
L-Cystine, 0.3%	.....	... ..	0/10
dl, $\alpha$ -Tocopheryl acetate, 40 mg/kg	...	.....	0/10

<sup>1</sup> Basal diet contains 4% cottonseed oil (Wesson oil) and 10 mg/kg of dl alpha-tocopheryl acetate.

<sup>2</sup> Basal diet contains 4% cottonseed oil, but no supplemental vitamin E.

<sup>3</sup> Numerator is the number of chicks showing grossly visible muscular degeneration (white striations); the denominator is the number of chicks examined in each group, which in almost all cases also represents the number of survivors.

In the first two experiments there was a high incidence of muscular degeneration in chickens fed the basal diet which was almost completely prevented by the addition of 0.3% of DL-methionine (table 1). Since this diet contains a substantial amount<sup>4</sup> of vitamin E (32 I.U./kg), it was first thought that degeneration was due to a simple methionine (or cystine) deficiency. However, the muscular degeneration did appear similar histologically to that reported for vitamin E-deficient animals (Pappenheimer, '43) and therefore the effect of a high level of vitamin E was tested in the third experiment. In this and several subsequent experiments, it was clearly de-

<sup>4</sup> Based on alpha-tocopheryl values in Harris et al. ('59).



monstrated that muscular degeneration could be completely prevented by an adequate dietary supplement of vitamin E. It would appear possible that the vitamin E found in cottonseed oil is not completely available to the chick or that the dietary requirement of vitamin E for the prevention of muscular degeneration is very high. The addition of 0.3% of L-cystine also completely prevented the lesion. When 0.5% of sodium sulfate was added there was a slight reduction in the incidence of muscular degeneration. All of these observations were confirmed in other experiments.

*Experiments with lard as the fat source.* In order to reduce the vitamin E level of the diet, lard was substituted for cottonseed oil. Dam et al., ('52) had found that several antioxidants including methylene blue were almost ineffective in the prevention of muscular degeneration. In experiments 4 and 5 DPPD was tested since this antioxidant is reported (Singsen et al., '55) to be effective in the prevention of encephalomalacia. When 0.05% DPPD was added, the incidence of muscular degeneration was not affected appreciably (table 2). However, at a level of 0.1% there was almost complete protection and subsequent experiments showed that higher levels would give complete protection. The addition of 40 mg/kg of the antioxidant Tenox R,<sup>5</sup> had no effect.

Removal of fat from the diet or the addition of tryptophan had no effect on the incidence or severity of muscular degeneration. The data in table 2 also demonstrate that the protective action of methionine is not due to any possible beneficial effects of increased food intake since birds fed a fat-free diet, or a diet containing Tenox-R consumed as much feed as those fed methionine and still had severe muscular degeneration.

Inositol had not been included in the basal diet since this compound is unnecessary for optimum growth of the chick (Machlin, '49). However, inositol was reported to counteract vitamin E deficiency symptoms in chicks (Dam, '44), and

<sup>5</sup> Tenox R, Eastman Chemical Products, Kingsport, Tenn., contains 20% butylated hydroxy anisole, 20% citric acid and 60% propylene glycol.

therefore the effect of this compound on muscular degeneration was tested. It was found that the addition of inositol to the diet had no effect on muscular degeneration (table 2). In this same experiment it was found that the use of a more purified type of casein was also ineffective.

TABLE 2

*Incidence and severity of muscular degeneration in chicks fed a diet containing lard<sup>1</sup>*

SUPPLEMENTS	INCIDENCE	SEVERITY <sup>2</sup>	FEED
			CONSUMPTION
			<i>gm/chick</i>
None	31/37	2.4	
dl, $\alpha$ -Tocopheryl acetate, 0.01%	0/25	..	
DL-Methionine, 0.5%	0/11	..	314
Tenox R, 40 mg/kg <sup>3</sup>	12/12	3.6	308
Glucose replaces lard	10/12	3.2	309
DL-Tryptophan, 0.1%	12/12	3.2	
Na <sub>2</sub> SO <sub>4</sub> , 0.5%	4/10	1.5	
DPPD, <sup>4</sup> 0.05%	9/12	2.0	
DPPD, 0.10%	1/11	3.0	
DPPD, 0.25%	0/13		
Inositol, 0.5%	11/13	2.1	
"Vitamin-free" casein	10/12	2.1	

<sup>1</sup> Basal contains 4% freshly rendered lard. The table is a summary of 4 experiments.

<sup>2</sup> Muscles of the breast for each bird scored as follows; 1 to 3 white striations visible 1; over 3 striations visible 2; entire breast area containing striations 3; most of breast area covered with striations most of which are more than 2 mm in width 4. The score for each experimental group is the average of those birds having any evidence of degeneration.

<sup>3</sup> Eastman Chemical Products, Kingsport, Tenn., contains 20% butylated hydroxy anisole, 20% citric acid, 60% propylene glycol.

<sup>4</sup> Diphenyl-p-phenylenediamine.

*Experiments with "stripped" lard.* In a final attempt to reduce the vitamin E level of the diet to a minimum and still have a source of essential fatty acids, a lard "stripped" of almost all vitamin E activity was used as the fat source in the remainder of the experiments.

It has been reported by Schwarz ('51) that the liver necrosis produced in rats fed diets deficient in vitamin E and sulfur

TABLE 3

*Growth and incidence of muscular degeneration of chicks fed diets containing "stripped" lard<sup>1</sup>*

SUPPLEMENTS	WEIGHT <i>gm at 4 weeks</i>	INCIDENCE
None	250	63/94
E <sup>2</sup>	245	0/21
Inositol, 0.5%	258	19/24
Thioctic acid, <sup>3</sup> 1 mg/kg	256	12/12
Thioctic acid, 2 mg/kg	271	3/12
Thioctic acid, 4 mg/kg	278	12/12
Thioctic acid, 8 mg/kg	233	11/13
E + 0.5% M <sup>2</sup>	349	0/12
E + 0.5% M + 0.5% inositol	357	0/12
E + 0.5% M + thioctic acid, 1 mg/kg	364	0/12
E + 0.5% M + thioctic acid, 4 mg/kg	355	0/12
Taurine, 0.5%	247	10/13
Taurine, 1.0%	241	11/13
Brewers' yeast, <sup>4</sup> 10%	259	15/25
Brewers' yeast, 20%	198	8/13
Brewers' yeast, 30%	144	5/13
DL-Methionine, 0.1%	294	10/12
DL-Methionine, 0.2%	306	5/13
DL-Methionine, 0.3%	322	1/13
L-Cystine, <sup>5</sup> 0.08%	290	7/13
L-Cystine, 0.16%	302	4/13
L-Cystine, 0.24%	315	0/13
DPPD, <sup>6</sup> 0.05%	237	6/11
DPPD, 0.10%	243	2/13
DPPD, 0.15%	238	2/8
DPPD, 0.25%	238	0/13
Chlorotetraeycline, 0.01%	290	8/10

<sup>1</sup> Basal contains 2% "stripped" lard. Distillation Products Industries, Rochester, N. Y. 500 mg/kg of Tenox R was added to the lard immediately after opening each can. The table is a summary of 5 experiments.

<sup>2</sup> E = 0.01% *dl* alpha tocopheryl acetate, M = DL-methionine.

<sup>3</sup> Furnished by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y.

<sup>4</sup> Brewers' yeast composite furnished by Elsie Singruen, Brewers' Yeast Council, Inc., Chicago, Ill., The yeast replaces equal parts of casein and glucose.

<sup>5</sup> Equivalent to comparable methionine on a sulfur basis.

<sup>6</sup> Diphenyl-*p*-phenylenediamine.

can be prevented by addition of a factor (Factor 3) present in brewers' yeast. Moreover Scott et al., ('55) found that the addition of brewers' yeast to a diet low in vitamin E prevented exudative diathesis. The data (table 3) demonstrated that brewers' yeast at levels as high as 30% did not prevent muscular degeneration. A sample of yeast was fed which was known to be effective in the prevention of exudative diathesis under the conditions employed by Scott et al. ('55).<sup>6</sup>

The data in table 3 also show that inositol and thioctic acid have no effect on muscular degeneration. Moreover they have no effect on growth when added to the basal diet or to diets containing vitamin E and methionine. Taurine, a sulfur-containing compound, was also without effect.

Chlorotetracycline, which was reported to aid in the prevention of liver necrosis in rats (Gyorgy et al., '51) had no effect on the development of muscular degeneration in the chicken.

Methionine and cystine appear to be approximately equal in protective activity. This is in contrast to the report (Schwarz, '54) that methionine is only 35% as active as cystine for the prevention of liver necrosis.

*Injection of DPPD.* In order to determine whether the site of the action of DPPD was in the intestinal tract or in the body of the chicken, DPPD was injected. A suspension of DPPD in 0.5% Tween-80 was injected into the leg muscle three times weekly so as to supply the equivalent of chicks receiving 0.01% or 0.05% DPPD in their ration. The antioxidant completely prevented muscular degeneration at the 0.05% level and only two out of 8 chicks were affected at the 0.01% level. It would therefore appear that the primary site of its function is in the tissues of the body.

#### DISCUSSION

Although muscular degeneration is not a common manifestation of a vitamin E deficiency in chickens (Pappenheimer, '43) it has been observed in ducks (Pappenheimer

<sup>6</sup> Scott, M. L., personal communication.

and Goettsch, '34; Gerriets, '54) and pheasants (Jungheer et al., '49). It appears however from the work of Dan et al. ('52), and that reported here, that muscular degeneration does occur in chickens if the diet is also deficient in sulfur. Since it is quite possible that the diets used in the studies with ducks were deficient in cystine and methionine it is possible that the difference in response to vitamin E-deficiency between these species is not as distinct as once thought.

Our work would indicate that the total requirement for methionine and cystine for the prevention of muscular degeneration is approximately the same as that required for optimum growth. The reduction in the incidence of muscular degeneration resulting from the addition of sodium sulfate is probably due to the action of sulfate in sparing the requirement of the chicken for the sulfur-containing amino acids (Gordon and Sizer, '55; Machlin, '55). The protective effect of cystine and methionine against muscular degeneration is in contrast to the lack of their effect on exudative diathesis (Dam et al., '51; Scott et al., '55), or of cystine or methionine on encephalomalacia (Singsen et al., '55).

The addition of brewers' yeast to the diet did not increase growth or affect muscular degeneration, whereas this supplement did increase growth and prevented exudative diathesis under the conditions employed by Scott et al. ('55). The reason for these differences is not clear, nor is there any obvious explanation for the fact that only muscular degeneration occurred in chicks fed diets low in vitamin E and sulfur, whereas exudative diathesis was observed by the Cornell workers in chicks fed diets deficient in these same nutrients. It is possible that the *Torula* yeast used by these workers is deficient in ingredients such as Factor 3 or available amino acids which are present in a casein-gelatin type of diet, or that this yeast contains dietary factors (e.g. fatty acids, purines, pyrimidines) which strongly influence the manifestation of a vitamin E deficiency.

The studies presented here show that the requirement for DPPD for the prevention of muscular degeneration is be-

tween 0.15% and 0.25% of the diet. This is much higher than the level of 0.025% found to be adequate for complete protection of encephalomalacia (Singsen et al., '55). DPPD was also effective when injected. Although DPPD might function by the protection of small amounts of vitamin E present in the basal diet or in the tissues of the bird, the studies of Singsen et al. ('55) on the effect of DPPD on blood tocopheryl suggest that a dietary level of much less than 0.15% would suffice for such a function. Until more is known about the mechanism of action of antioxidants, it will be difficult to explain how DPPD can prevent muscular degeneration or why there is a higher level of this antioxidant needed for the prevention of muscular degeneration compared to the requirement for protection against encephalomalacia.

#### SUMMARY

Chickens fed diets low in vitamin E and sulfur to 4 weeks of age developed a muscular degeneration manifested grossly as white striations of the breast and leg muscles, and microscopically as a hyaline type degeneration. No significant changes were noted in the tissues of the gizzard, heart, liver, pancreas, kidney or spleen.

The addition of alpha tocopheryl-acetate, methionine, cystine, or a high level of diphenyl-p-phenylenediamine (DPPD) (0.25%) to the diets completely prevented muscular degeneration. Addition of 0.5% of sodium sulfate slightly reduced the incidence of muscular degeneration. However supplements of brewers' yeast, tryptophan, chlorotetracycline, inositol, taurine or thioctic acid had no effect on this pathological alteration.

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A STUDY OF THE EFFECT OF CHLORTETRACYCLINE (AUREOMYCIN) UPON CALCIUM RETENTION BY THE GROWING MALE ALBINO RAT<sup>1</sup>

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Numerous theories have been advanced to explain the stimulation of animal growth given by antibiotics. One of the more prominent theories suggests that the antibiotic induces increased absorption of nutrients from the gastrointestinal tract. If this were the main mode of action, it would be expected that mineral absorption would be increased and a greater amount of ingested calcium (Ca) would be available to the animal for the laying down of bone tissue. Migicovsky et al. ('51) reported that dietary penicillin significantly increased the Ca absorption index of chicks which had previously been fed a low Ca diet. Ross and Yacowitz ('54) found that dietary procaine penicillin G significantly increased chick bone (tibia) ash. Rusoff et al. ('54) reported larger skeletal growth and increased bone size in young calves to which aureomycin was administered parenterally or orally. Murray and Campbell ('55) found the response of rachitic rats to doses of vitamin D, as measured by the line test (U.S.P. XIV, '50), was increased by the addition of aureomycin to the diet.

The present study was undertaken to test whether aureomycin could, during a 5-week growth period, increase the re-

<sup>1</sup> Authorized on 3/7/56 for publication as paper no. 2059 in the *Journal Series* of the Pennsylvania Agricultural Experiment Station.



tention of carcass calcium in weanling rats given experimental diets containing two levels of Ca (0.55% and approximately one-half of this amount) in the presence of 0.44% of inorganic phosphorus and adequate vitamin D under the condition of equal food intakes within replicates of animals.

#### EXPERIMENTAL

Forty weanling male albino rats weighing 42 to 64 gm and housed in individual screen-bottom cages were assigned on the basis of body weight and litter to 10 replicates of 4 animals each. All rats within a replicate received daily quantities of experimental diets equal to the consumption of the smallest eater.<sup>2</sup> Rat 1 of each replicate (*basal*) received a ration containing, in percentage, vitamin-test casein,<sup>3</sup> 18; DL-methionine, 0.2; corn oil,<sup>4</sup> 7; cornstarch, 40; sucrose, 10; cerelese, 18.68; vitamin mix,<sup>5</sup> 0.5; choline chloride, 0.1; cellulose, 2; modified Salts IV (Ca-free),<sup>6</sup> 2.85; and CaCO<sub>3</sub>, 0.67. Rat 2 of each replicate (*basal* + *aureomycin*) received a diet identical to that given rat 1 to which aureomycin had been thoroughly admixed at the rate of 100 mg/kg of diet. Rat 3 of each replicate (*basal* + *Ca*) received a diet the same as that given rat 1 except that 4% of Salts IV<sup>6</sup> replaced the modified Salts IV and CaCO<sub>3</sub>. Rat 4 of each replicate (*basal* + *aureomycin* + *Ca*) received a diet identical to that given rat 2 to which aureomycin had been thoroughly admixed at the rate of 100 mg/kg of diet. The additional minerals of the latter two diets were added at the expense of cerelese. By analysis

<sup>2</sup> It was long ago pointed out by Fairbanks and Mitchell ('36) that "the control of food intake in calcium metabolism studies is essential to the most significant results."

<sup>3</sup> Nutritional Biochemicals Corporation, Cleveland, Ohio.

<sup>4</sup> Oleum Percomorphum (Mead Johnson & Co., Evansville, Indiana) and  $\alpha$ -tocopheryl acetate added to the corn oil so that the final diet contained 2,000 I.U. vitamin A 283 I.U. vitamin D, and 10 mg vitamin E per 100 gm.

<sup>5</sup> The vitamin mix had the following composition: thiamine hydrochloride 500 mg, riboflavin 600 mg, pyridoxine hydrochloride 300 mg, Ca pantothenate 3 gm, nicotinic acid 6 gm, folic acid 100 mg, vitamin K 100 mg, vitamin B<sub>12</sub> 5 mg, biotin 10 mg, and cerelese to make 500 gm total.

<sup>6</sup> Lichstein et al. ('46).

the above diets contained in the order given 0.291, 0.286, 0.549, and 0.551% Ca. Additional vitamin B<sub>1</sub> (50 mg/rat/day as a cerelese premix) was added to each daily portion of diet. This was done in order to replace possible losses of the vitamin by components of the salt mixture, as shown by Waibel et al. ('54). Distilled water was given to all rats ad libitum.

After 35 days on the experiment the animals were killed with chloroform, their body lengths from tip of nose to base of tail were measured, the gastrointestinal tracts were emptied, the carcasses weighed, cut into small pieces and dried in vacuum desiccators over concentrated H<sub>2</sub>SO<sub>4</sub> for approximately 10 days. The fat was removed from the dry carcasses by 48 hours extraction with diethyl ether in a Soxhlet apparatus and the dry residue was finely ground in a burr mill. Thus carcass fat and dry matter were measured directly, but carcass water was determined indirectly. Calcium was determined on a 5 gm sample of the finely ground dry residue by the A.O.A.C. ('50) method. Eight litter-mate rats were killed at the beginning of the experiment and were dried, extracted, ground and analyzed in an identical manner.

#### RESULTS AND DISCUSSION

Table 1 summarizes the experimental results obtained, showing group means and the standard error of the means for the various criteria used to evaluate the experiment. Column 1 of the table contains the items tested: body weight gain ("empty" carcass weight — initial body weight); carcass dry matter gain, carcass water gain, and carcass ether extract gain (respectively, the content of these substances in the "empty" carcass — the initial amounts present); carcass Ca gain (Ca content of the "empty" carcass — initial Ca); and percentage Ca retention [(carcass Ca gain/Ca intake) × 100]. In order to arrive at initial dry matter, water, ether extract, and Ca values, initial body weights were multiplied by the mean percentages (20.4, 66, 3.99, and .85, respectively) found for these substances in the carcasses of the control rats killed at the beginning of the experiment. Columns 2, 3, 4, and

5 show mean values obtained for groups 1, 2, 3, and 4, respectively. Column 6 shows the standard errors of the means. The number of animals in each group was originally 10, but the data given in table 1 cover, in some cases, smaller numbers. In one instance an animal escaped from its cage for approximately 12 hours and was removed from the experiment; another animal died from unknown cause and was discarded;

TABLE 1  
*Effect of aureomycin on growth and calcium content of the body*

ITEM	GROUP 1	GROUP 2	GROUP 3	GROUP 4	STANDARD ERROR OF THE MEANS
	Basal (9) <sup>1</sup>	Basal + aureo- mycin (10) <sup>1</sup>	Basal + Ca (9) <sup>1</sup>	Basal + aureo- mycin + Ca (10) <sup>1</sup>	
	<i>mean</i>	<i>mean</i>	<i>mean</i>	<i>mean</i>	
Body length, cm	21.42	21.33 <sup>2</sup>	21.38	21.27	± 0.15
Body weight gain, gm	180.9	180.8	176.8	175.1	± 6.3
Carcass dry matter gain, gm	44.51 <sup>2</sup>	42.81 <sup>2</sup>	43.27	41.89	± 1.44
Carcass water gain, gm	114.78 <sup>2</sup>	111.59 <sup>2</sup>	106.94	102.64	± 3.48
Carcass ether extract gain, gm	31.98 <sup>2</sup>	34.84 <sup>2</sup>	31.61	35.61	± 2.38
Carcass Ca gain, gm	1.092	1.095	1.356	1.323	± 0.045
Ca retention, %	77.3	79.1	51.7	49.7	± 2.0

<sup>1</sup> Number of animals in group.

<sup>2</sup> Number of animals = 8.

<sup>3</sup> Number of animals = 9.

in another instance the body length measurement was inadvertently not recorded; and finally, data for ether extract gain of two animals are not included because there was evidence that an undetermined loss of carcass fat had occurred during the ether extraction.

Analyses of variance were made for the criteria of response given in column 1 of table 1. The interactions of additional dietary Ca with the antibiotic were small and failed in each

case to attain statistical significance. For this reason the variance of the main effects of the treatments and the probability that such variance resulted from chance alone was determined. The results of such statistical treatment appear in table 2. For convenience, the mean values for the control group appear in column 2, and the mean deviations from these values caused by the main effects and interaction, appear in columns 3, 4, and 5, respectively. The main effects

TABLE 2

*Main effects and interactions of added calcium and aureomycin*

ITEM	CONTROL GROUP	MEAN MAIN EFFECTS		MEAN INTER- ACTION
	Mean	Calcium	Aureomycin	
Body length, cm	21.42	— 0.05	— 0.10	— 0.02
Body weight gain, gm	180.9	— 4.9 <sup>1</sup>	— 0.9	— 1.6
Carcass dry matter gain, gm	44.51	— 1.08	— 1.54 <sup>1</sup>	0.32
Carcass water gain, gm	114.78	— 8.39 <sup>2</sup>	— 3.74	— 1.11
Carcass ether extract gain, gm	31.98	0.20	3.43 <sup>2</sup>	1.14
Carcass Ca gain, gm	1.092	0.246 <sup>2</sup>	— 0.015	— 0.036
Ca retention, %	77.3	— 27.5 <sup>2</sup>	— 0.1	— 3.8

<sup>1</sup>  $P < 0.05$ .

<sup>2</sup>  $P < 0.01$ .

<sup>3</sup>  $P \approx 0.051$ .

of additional dietary Ca were a significant decrease in body weight gain, a highly significant decrease of carcass water gain, and a highly significant increase of the carcass Ca gain — the latter accompanied by a highly significant decrease in the percentage of Ca retention. The finding of an increased carcass Ca gain accompanied by a decreased percentage of retention when an additional increment of dietary Ca was given is in accord with the previous experience of Fairbanks and Mitchell ('36). The finding of a significant decrease in

body weight gain with the increment of dietary Ca added is largely explained by the highly significant decrease in carcass water gain and is also in accord with the findings of the previously cited authors, since they found that an inverse relation exists between the rate of growth and the Ca content of the carcass.

The main effects of aureomycin addition to the basal diet were slight and non-significant decreases in all of the criteria tested save carcass dry matter gain and carcass ether extract gain. In regard to the former criterion a significant decrease was noted. However, in the case of the latter criterion, the increase of 3.43 gm was indeed close to statistical significance. The tendency toward increased deposition of fat incident to the feeding of antibiotics is in agreement with the work of Black and Bratzler ('52), who used both a crude vitamin B<sub>12</sub> supplement containing streptomycin and streptomycin without added B<sub>12</sub>; with the work of Knoebel and Black ('52), who used a similar crude vitamin B<sub>12</sub> supplement containing streptomycin and terramycin together with additional terramycin and added aureomycin; with the work of Hartsook and Johnson ('53), who used terramycin, but at variance with the work of Forbes ('54), who used streptomycin and chloromycetin. In the references cited, however, it appears that the amount of carcass ether extract gain obtained upon feeding antibiotic (s) stands in inverse relationship to the adequacy of the diets for animal growth.

The finding that increased utilization of Ca over the entire experimental period did not occur in this work with the rat, on supplying aureomycin, as was found in short-term experiments with chicks (Migicovsky et al., '51) and that increased skeletal growth as would be indicated by increased body length was not found in this work as was reported in work with cattle (Rusoff et al., '54) can probably be explained by species differences in response to antibiotics. Species differences in growth responses to antibiotics have been repeatedly noted.

## SUMMARY

Ten replicates of 4 weanling, male, albino rats each were fed daily for a 35-day period equal quantities of the following experimental diets: rat 1 received the basal diet (containing approximately 18% casein, 0.29% Ca and 0.44% P), rat 2 received the basal diet + aureomycin (100 mg/kg), rat 3 received the basal diet + additional Ca (0.55% Ca), and rat 4 received the basal diet + additional Ca and aureomycin.

Calcium supplementation of the basal diet resulted in a highly significant increase (22.5%) in carcass Ca gain, a significant decrease (2.7%) in body weight gain, a highly significant decrease (7.3% and 35.6%, respectively) in carcass water gain and in the percentage of Ca retention.

Aureomycin supplementation of the basal diet did not increase body weight gain, carcass water gain, body length, carcass Ca gain, or the percentage of Ca retention. Aureomycin, did, however, increase (10.7%) the carcass ether extract gain to an extent that closely approached statistical significance at the 5% level, and significantly decreased (3.5%) carcass dry matter gain.

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# THE PEPSIN-DIGEST-RESIDUE (PDR) AMINO ACID INDEX OF NET PROTEIN UTILIZATION<sup>1</sup>

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Although the nutritional quality of a protein must in the final analysis be established biologically there are many advantages to be derived from an *in vitro* method which accurately predicts the biological value of a protein from its chemical composition. It has been recognized since the investigations of Osborne and Mendel that the nutritional value of a protein is primarily dependent upon its constituent amino acids. Mendel ('23) proposed that the efficiency of the individual protein in this respect must depend on the minimum quantity of any indispensable amino acid that it will yield. On the basis of this concept Mitchell and Block ('46) evolved a workable system for the quantitative evaluation of a protein from its amino acid composition. Using whole egg protein as the standard, the nutritive value of a protein was expressed as a "chemical score," equal to the greatest percentage deficit in an essential amino acid in the test protein. Oser ('51) devised a similar method in which all of the essential amino acid "egg ratios" were integrated by calculation of their geometric mean. With reference to man and the rat both methods of scoring yielded values which correlate well with the biological values<sup>2</sup> of a variety of

<sup>1</sup> A preliminary report of this work was presented before the American Society of Biological Chemists at San Francisco, California, April 11-15, 1955 (Sheffner, Eckfeldt and Spector, '55).

<sup>2</sup> The term biological value is used in this paper in accordance with the formula introduced by Mitchell ('24) after Thomas.



proteins. However, for many proteins, the calculated values did not reflect the quantitative differences in biological value.

Mitchell ('52), while reviewing a half-century of progress in nutritional evaluation of proteins questioned whether a method which depended in its entirety upon the total amino acid composition could predict precisely the biological value of proteins, since many other factors affected the utilization of dietary protein. One of these factors is undoubtedly related to the observation that delayed supplementation of a deficient protein with the lacking amino acids is ineffective in correcting the deficiency (Berg and Rose, '29; Geiger, '47; Henry and Kon, '46). Based upon this fact and upon subsequent work of their own Melnick and co-workers ('46) and Riesen et al. ('47a) proposed that in addition to the total amino acid composition, the rate of release of amino acids from protein by pancreatic digestion was also an important factor in the nutritional quality of a protein. This concept was utilized by Horn et al. ('52) to evaluate the nutritional quality of food proteins by measuring microbiologically the individual amino acids made available by pepsin, trypsin, and hog mucosa. This method gave good correlation with the biological value of cottonseed meal which had been subjected to various degrees of processing; however, there was no indication that it could be used to compare proteins from different sources. Halevy and Grosowicz ('43), using the growth response of *Streptococcus fecalis* to a pancreatic digest of the test protein, and Dunn and Rockland ('47) and Anderson and Williams ('51), using the proteolytic protozoan, *Tetrahymena geleii*, also developed procedures to estimate the biological value of proteins. However, the values obtained with these methods did not correlate well with the biological value of proteins as determined by rat assay.

It was the purpose of the present investigation (1) to determine the relationship between the biological value of food proteins and the patterns of amino acids released by digestive enzymes, and (2) to develop an *in vitro* procedure which could accurately estimate the nutritional value of proteins.

## METHODS AND MATERIALS

Pepsin digests were prepared by incubating 1 gm of protein ( $N \times 6.25$ ) with 25 mg of pepsin (U.S.P. 1:10,000) <sup>3</sup> in 30 ml of 0.1 N  $H_2SO_4$  for 24 hours at 37°C. For pepsin plus trypsin digests, the pepsin digests were buffered with 3 gm of  $KH_2PO_4$ , adjusted to pH 8.4, and incubated with 25 mg of trypsin ( $4 \times$  U.S.P. pancreatin),<sup>3</sup> at 37°C. for 24 hours. For subsequent erepsin digestion, samples previously treated with pepsin and trypsin were adjusted to pH 7.8 and incubated with 100 mg of erepsin <sup>3</sup> at 37°C. for 72 hours. The samples were covered with toluene during incubation and stirred occasionally. Enzyme blanks were prepared for each stage of digestion with 10 times the quantity of enzyme used for sample treatment. At the end of the indicated incubation periods the digests were heated in a boiling water bath for 10 minutes, cooled and adjusted to pH 2. One volume of 10% sodium tungstate and one volume of  $2/3$  N  $H_2SO_4$  were then added for each 8 volumes of digest. The mixtures were allowed to stand for 10 minutes and filtered. The clear filtrates were adjusted to pH 6.8, diluted to the appropriate amino acid concentration, and stored frozen until analyzed. In the case of starchy samples, e.g., white flour, most of the starch was removed by centrifugation before the digest was heated.

Acid hydrolysates for the determination of the total amino acid content were prepared by autoclaving each protein for 16 hours at 120°C. with 20 ml of 2 N HCl per gram dry weight of sample. However, for the measurement of cystine the samples were autoclaved only three hours (Riesen et al., '47b). Alkaline hydrolysates for the assay of tryptophan and tyrosine were prepared using 20 ml of 5 N NaOH per gram dry weight of sample and autoclaving at 120°C. for 5 hours. To eliminate gel formation in the flour samples these were steamed for 30 minutes in 25 ml of 0.1 N HCl prior to hydrolysis with alkali. Individual amino acid analyses were, in general, per-

<sup>3</sup> Nutritional Biochemicals Corporation, Cleveland, Ohio.

formed by microbiological procedures previously described (Sheffner et al., '48); however, the final volume per tube was 2 ml and the acid produced by the growth of the organisms was measured by means of a quinhydrone electrode and the Cannon titration apparatus. Also, for the determination of cystine the medium and samples were autoclaved separately to minimize destruction of this amino acid. *Leuconostoc mesenteroides* P-60 was used for the assay of cystine, histidine,

TABLE 1  
"Essential" amino acids in whole egg and casein

AMINO ACID	WHOLE EGG			CASEIN		
	Total	Pepsin digest <sup>1</sup>	Residue	Total	Pepsin digest <sup>1</sup>	Residue
		mg/gm			mg/gm	
Histidine	26.8	0.45	26.4	31.8	0.07	31.7
Lysine	82.8	2.9	79.9	80.8	0.34	80.5
Methionine	31.6	7.2	24.4	30.4	0.16	30.2
Cystine	21.4	0.54	20.9	3.4	0.07	3.3
Phenylalanine	55.4	11.4	44.0	55.4	4.8	50.6
Tyrosine	36.3	5.9	30.4	52.0	6.2	45.8
Leucine	80.7	52.5	28.2	98.4	38.2	60.2
Isoleucine	55.4	38.2	17.2	58.1	5.9	52.2
Valine	66.3	11.7	54.6	72.5	1.1	71.4
Threonine	49.0	25.9	23.1	41.2	12.5	28.7
Tryptophan	13.5	6.8	6.7	11.6	4.0	7.6
	519.2	163.5	355.8		73.3	462.2

<sup>1</sup> Corrected for enzyme blank.

lysine, methionine, phenylalanine, tyrosine and valine, and *Streptococcus fecalis* for isoleucine, leucine, threonine and tryptophan. Total nitrogen was measured by the macro-Kjeldahl procedure using mercuric oxide as the digestion catalyst. Alpha amino nitrogen was determined by the copper titration method of Pope and Stevens ('39).

#### CALCULATION OF THE PDR INDEX

The 8 amino acids essential to human nutrition plus cystine and tyrosine are used when the test protein is evaluated for

man. When the protein is evaluated for the growing rat, histidine is also taken into account. To illustrate the method, calculation will be made of the PDR index of casein for use with the growing rat.

The concentrations of these 11 amino acids in whole egg and in casein are determined in the completely hydrolyzed proteins and in the pepsin digests. The concentration of each amino acid (mg/gm of protein) microbiologically available in the pepsin digest is subtracted from the concentration of the respective amino acid in the total hydrolysate to give the "residue" fraction (table 1).

Each amino acid is then calculated as the percentage of the sum of the 11 amino acids for the protein in the pepsin digest and residue fractions, respectively. For example, the "free" histidine content of the pepsin digest of whole egg is 0.45 mg. The sum of the 11 amino acids in the pepsin digest is 163.5 mg. Histidine thus constitutes  $0.45/163.5$  or 0.28% of the sum of these 11 amino acids in the pepsin digest (table 2).

The ratio of the percentage of each amino acid in the pepsin digest of casein to the percentage of that amino acid in the pepsin digest of whole egg gives the "egg ratio." The geometric mean of the adjusted egg ratios is then computed logarithmically by averaging the logarithms of the egg ratios, and obtaining the antilogarithm. A similar calculation is made for the residue fraction. Egg ratios less than one are considered to be one in order to avoid negative logarithms. In computing the egg ratios, percentage concentrations of amino acids in excess of those present in the standard protein are disregarded. Methionine and cystine are considered as a unit, as are phenylalanine plus tyrosine. If the essential precursor amino acid of the pair, e.g., methionine, is present in excess of that in egg, the excess can be used to make up the deficiency of the non-essential amino acid, but the reverse is not done. For example, the pepsin digest of casein is credited with only 3.61% of tyrosine, i.e., the same as in whole egg, rather than 8.46%. The total of phenylalanine (6.55%)

plus tyrosine (3.61%) is thus 10.16% and the egg ratio is 10.16/10.58 or 96.0.

The geometric means of the fractions are each multiplied by a factor to correct for the degree of proteolysis of the test protein relative to that for the standard egg protein. The

TABLE 2  
*Example for calculation of the PDR index for casein*

AMINO ACID	PEPSIN DIGEST				RESIDUE			
	Whole egg	Cas.	Egg ratio	Log egg ratio	Whole egg	Cas.	Egg ratio	Log egg ratio
	%	%			%	%		
Histidine	0.28	0.10	35.7	1.5527	7.42	6.86	92.5	1.9661
Lysine	1.77	0.46	26.0	1.4150	22.46	17.42	77.6	1.8899
Methionine	4.40	0.22			6.86	6.53		
Cystine	0.33	0.10			5.87	0.71		
Meth. and cys.	4.73	0.32	6.8	0.8325	12.73	7.24	56.9	1.7551
Phenylalanine	6.97	6.55			12.37	10.95		
Tyrosine	3.61	8.46			8.54	9.91		
Phen. and tyr.	10.58	10.16	96.0	1.9823	20.91	19.49	93.2	1.9694
Leucine	32.11	52.11	100.0	2.0000	7.92	13.02	100.0	2.0000
Isoleucine	23.36	8.05	34.5	1.5378	4.83	11.29	100.0	2.0000
Valine	7.16	1.50	20.9	1.3201	15.34	15.45	100.0	2.0000
Threonine	15.84	17.05	100.0	2.0000	6.49	6.21	95.7	1.9809
Tryptophan	4.16	5.46	100.0	2.0000	1.88	1.64	87.2	1.9405

Av. logarithm of egg ratios = 1.6267 1.9446

Geometric mean:

Pepsin digest fraction = antilog 1.6267  
= 42.3

Residue fraction

= antilog 1.9446  
= 88.0

Corrected geometric mean:

Pepsin digest fraction =  $42.3 \times 73.3/163.5$   
= 18.96

Residue fraction

=  $88.0 \times 462.2/355.8$   
= 114.32

PDR index =  $\text{antilog } (0.315 \times \log 18.96 + 0.685 \times \log 114.32) = 64.9$

PDR index/digestibility =  $64.9/0.97 = 66.9$

factor for the pepsin digest is obtained by summing the concentrations (mg/gm) of the 11 individual amino acids in the pepsin digest of the test protein and dividing the total by the sum of the concentrations obtained for the standard egg. For example, the sum of the 11 amino acids in the pepsin

digest of casein is 73.3 and the sum for egg is 163.5. The factor is 73.3/163.5. Similarly, for the residue fraction the factor is 462.2 (casein)/355.8 (egg). Multiplying the geometric mean of the two fractions by their respective proteolysis factors yields the corrected geometric means.

To obtain an amino acid index for the whole protein (casein) the corrected geometric means of the pepsin digest fraction and the residue fraction must be weighted in accordance with

TABLE 3

*Comparison of the patterns<sup>1</sup> of the microbiologically available amino acids in enzymatic digests and in completely hydrolyzed whole egg protein and casein*

AMINO ACID	WHOLE EGG				CASEIN			
	Pepsin	Pepsin + trypsin	Pepsin + trypsin + chymotrypsin	Total	Pepsin	Pepsin + trypsin	Pepsin + trypsin + chymotrypsin	Total
	%	%	%	%	%	%	%	%
Leucine	33.4	24.6	21.2	17.5	57.0	28.4	25.2	20.5
Isoleucine	24.3	13.6	12.1	12.0	8.8	11.2	10.1	12.1
Threonine	16.5	14.4	13.0	10.6	18.6	13.6	12.8	8.6
Valine	7.4	12.9	14.7	14.4	1.6	8.2	11.5	15.1
Phenylalanine	7.3	11.6	11.3	12.0	7.2	9.1	10.8	11.5
Methionine	4.6	5.6	7.0	6.8	0.2	2.9	3.0	6.3
Tryptophan	4.3	3.9	3.5	2.9	6.0	3.1	3.2	2.4
Lysine	1.8	10.4	13.6	17.9	0.5	21.1	20.9	16.8
Histidine	0.3	3.0	3.6	5.8	0.1	2.3	2.5	6.6

<sup>1</sup> Each amino acid value was calculated as the percentage of the sum of the 9 amino acids indicated.

the percentage each represents of the total standard egg protein. The concentrations of the 11 amino acids measured in the pepsin digest of the standard egg protein represented, on the average, 163.5/519.2 or 31.5% of these amino acids in the entire egg protein, and the amino acids in the residue represented 355.8/519.2 or 68.5% of the 11 amino acids in the entire egg protein. The corrected geometric means are weighted and averaged geometrically to obtain the PDR index for the whole protein.

## RESULTS

The patterns of microbiologically available essential amino acids present in enzymatic digests and in complete hydrolysates of whole egg and casein are given in table 3. It can be seen that there are large differences in the proportions of amino acids liberated from the two proteins by pepsin digestion. These differences are considerably reduced when digestion is continued by treatment with trypsin, or trypsin and erepsin. For example, isoleucine represented 24.3% of the 9 amino acids measured in the pepsin digest of whole egg, whereas it was only 8.8% in the pepsin digest of casein. This ratio of 3:1 was reduced to almost 1:1 following treatment with trypsin. Similarly, the proportion of methionine in the pepsin digest of whole egg was 23 times as great as in the pepsin digest of casein. After tryptic action the ratio is diminished by only about 2:1. Leucine, which is present in much smaller proportion in the pepsin digest of whole egg than of casein, is present in almost equal proportions in the tryptic digests of both proteins. When the proteins are hydrolyzed to completion the proportions of the 9 amino acids in the two hydrolysates are almost identical.

Melnick et al. ('46) have reported that similar differences in the rate of release of individual amino acids occur after treatment of proteins with pancreatin; however, the results presented here demonstrate that where proteins are first treated with pepsin, as occurs under physiological conditions, the differences between proteins upon subsequent treatment with trypsin are considerably reduced. These data prompted the hypothesis that if appreciable absorption of the products of peptic digestion occurred, some of the differences in biological value between proteins of comparable total amino acid content could be accounted for by the diversity in pattern of amino acids released by pepsin digestion.

If whole egg protein is considered as the standard, the total essential amino acid patterns in proteins can be compared by computing "egg ratios" (Oser, '51). The integrated essential amino acid egg ratios of a variety of proteins are presented

in table 4. These values are calculated according to the method of Oser ('51), as modified by Mitchell ('54) to include tyrosine but omit arginine in computing the revised index (modified essential amino acid index, MEAA). For many proteins, this total amino acid index does not reflect the actual biological value of the protein. For instance, the MEAA index suggests

TABLE 4

*Comparison of the PDR index of food proteins with their biological value and net utilization for the growing rat*

PROTEIN <sup>1</sup>	BIOLOGICAL VALUE	DIGESTI- BILITY	NET UTIL- IZATION	PDR INDEX	PDR/ DIGESTI- BILITY	MEAA INDEX	CHEMICAL SCORE
Whole egg	98 <sup>2</sup>	99	97	100	101	100	100
Egg albumin	97 <sup>2</sup>	100	97	95	95	96	84
Defatted egg (com'l)	87 <sup>2</sup>	97	84	83	86	93	75
Lactalbumin	84 <sup>2</sup>	98	82	82	84	92	70
Soy flour	75 <sup>2</sup>	96	72	71	74	82	44
Casein, Labeo	68 <sup>2,3,4,5</sup>	97	66	65	67	92	64
Brewers' yeast	66 <sup>2,6</sup>	93	61	61	66	72	36
White flour	52 <sup>2</sup>	100	52	51	51	65	26

<sup>1</sup> The test proteins used in this study were obtained from the following sources: dried whole egg, Blue Star Foods Co.; egg albumin, Emulsol Corp.; defatted egg, Viobin Corp.; lactalbumin (Labeo), Borden Co.; soy flour (Nutri Soy), Archer-Daniels-Midland Co.; casein (Labeo, vitamin free), Borden Co.; brewers' yeast, (U.S.P. XII), Nutritional Biochemicals Corp.; and white flour (Pillsbury's Best) Pillsbury Mills, Inc.

<sup>2</sup> Mitchell and Beadles ('50).

<sup>3</sup> Mitchell and Block ('46).

<sup>4</sup> Greaves, Morgan and Loveen ('38).

<sup>5</sup> Chick, Boas-Fixsen, Hutchinson and Jackson ('35).

<sup>6</sup> Mitchell ('48).

that casein and egg albumin have similar biological values; however, it is well established that egg albumin has a considerably higher biological value for the growing rat. Lactalbumin has a much lower biological value than egg albumin; but this is not apparent from the MEAA index.

In general, the MEAA index overestimates the biological value. This fact lends support to the hypothesis that much of the potential nutritional value of many proteins is lost



by the release and absorption of disproportionate amounts of essential amino acids at an early stage in digestion. Consequently, a new amino acid index was devised which takes into account the physiological availability of amino acids during digestion. This index combines the pattern of essential amino acids obtained from analysis of the pepsin digest with the amino acid pattern of the remainder of the protein to produce an integrated index—the Pepsin Digest-Residue (PDR) amino acid index.

TABLE 5

*Comparison of the PDR index of food proteins with their biological value and net utilization for adult man*

PROTEIN	BIOLOGICAL VALUE	DIGESTI- BILITY	NET UTIL- IZATION	PDR INDEX	PDR/ DIGESTI- BILITY	MEAA INDEX	CHEMICAL SCORE
Whole egg	100 <sup>1,2</sup>	96	96	100	104	100	100
Egg albumin	91 <sup>2</sup>	101	92	95	94	96	84
Soy flour	73 <sup>4</sup>	90	66	69	77	81	44
Casein, Labco	68 <sup>2</sup>	96	65	63	66	90	64
White flour	41 <sup>4</sup>	97	40	49	51	64	26

<sup>1</sup> Murlin et al. ('44).

<sup>2</sup> Murlin et al. ('48).

<sup>3</sup> Hawley et al. ('48).

<sup>4</sup> Bricker et al. ('45).

Comparison of the PDR amino acid index with the net protein utilization and the biological value of food proteins for the growing rat and for man is presented in tables 2 and 3. Values for the chemical score and the modified essential amino acid index of Mitchell (MEAA) are also given for comparison. It will be noted that the chemical score, based upon the limiting amino acid always underestimates the biological value. While the MEAA index gives values in closer agreement with the biological value, there are many cases where the correlation for the MEAA index is also poor. The PDR indices are found to be in close agreement with the net utilization values of the respective proteins for the growing rat and for man. Therefore, the values obtained by dividing the PDR index by digestibility are closely correlated with the biological

values. The regression lines correlating the biological values with the MEAA indices and the PDR/digestibility values are shown in figure 1. The correlation coefficient,  $r$ , of the regression line for the PDR index/digestibility is only slightly better

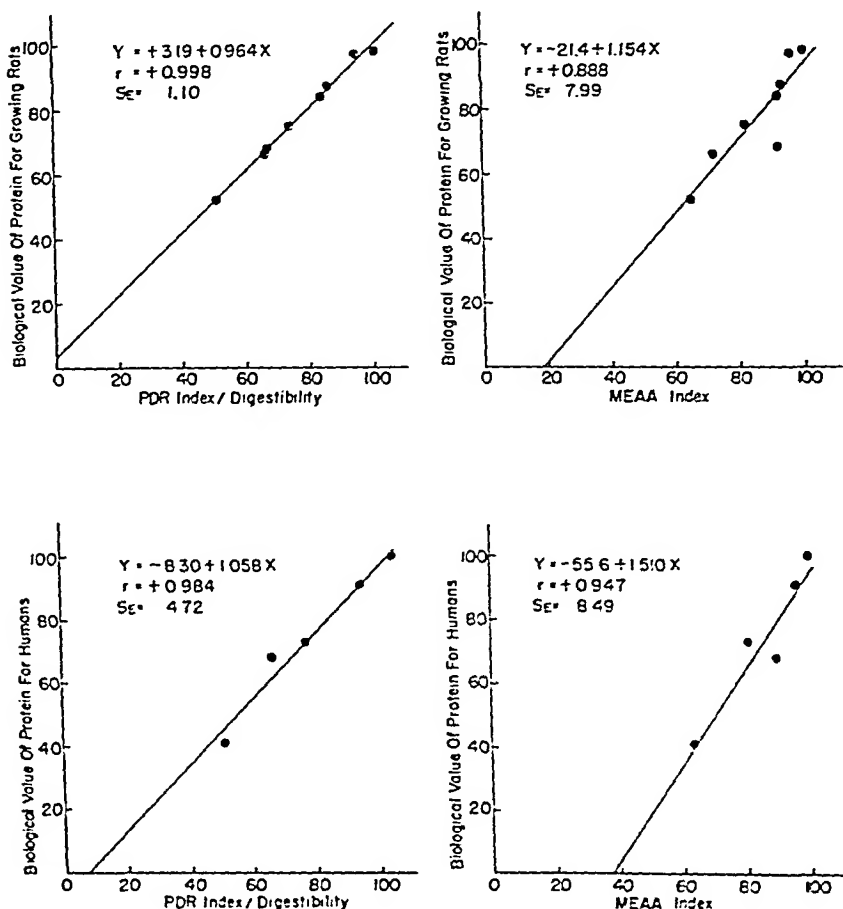


Fig. 1 Relationship between the biological values and (1) the PDR index/digestibility values and (2) the MEAA indices.

than that for the MEAA index since the latter index does, in general, show the proper order of value. However, the regression line for the PDR index/digestibility passes closer to the origin and shows a much smaller standard error of estimate,

$S_E$ , indicating that this index more accurately reflects the quantitative differences in biological value between the various proteins.

#### DISCUSSION

Following the proposal of Mitchell and Block ('46) whole egg protein was used as the reference protein for the PDR index of net protein utilization. These investigators reported that whole egg protein was almost perfectly utilized in digestion and metabolism for the growing rat. It was demonstrated in the present work that when the well-utilized standard egg protein was treated with digestive enzymes in a manner simulating the physiological sequence, then the pattern of amino acids changed as digestion proceeded. These results suggest that the optimal pattern varies with the stage of digestion or position in the digestive tract. In this respect, many investigators have found that whole protein is better utilized in the diet than protein hydrolysates or amino acid mixtures containing essentially equal quantities of amino acids (Woolley, '46; Womack and Rose, '46; Sheffner, Kirsner and Palmer, '50; Maddy and Swift, '52). Therefore, it appears that the standard of protein quality cannot be a non-varying pattern of amino acids.

The conditions established for obtaining *in vitro* pepsin digests were determined from feeding experiments with rats which indicated that approximately 30% of ingested egg protein nitrogen is absorbed before the chyme has reached the area of the intestine where tryptic activity is significant. Consequently, the quantity of pepsin used and the duration of incubation in the *in vitro* procedure were adjusted to produce approximately 30% release of microbiologically available essential amino acids from egg protein. The quantity of amino nitrogen released, when 1 gm of egg protein is digested with 25 mg of pepsin for 24 hours is almost 90% of that which occurs when either the amount of pepsin or the incubation time is doubled. With pepsin of the proper activity the conditions are such that small variations in time, temperature, and quantity of enzyme will not cause significant variation in

the PDR index. The activity of the pepsin used in these experiments was U.S.P. 1-10,000. Use of  $3 \times$  crystallized pepsin<sup>4</sup> resulted in a more rapid release of amino acids but did not change the pattern of amino acids made available.

The excellent correlation between the PDR index and the net protein utilization value of representative proteins suggested that this index should be a valuable *in vitro* method for predicting the nutritional quality of proteins. However, aside from this practical aspect the observed correlations suggest the importance of peptic digestion for optimal utilization of dietary proteins. In this respect, Mellander ('55), Kotschneff ('26, '28), and Sheffner et al. ('50) have suggested that the wall of the intestine is permeable to amino acid complexes. Consequently, it appears reasonable to suggest that an appreciable quantity of the products of pepsin digestion are absorbed from the duodenum before pancreatic enzymes can act on them, and that the pattern of these absorbed amino acids and peptides is an important factor in the efficient utilization of ingested protein.

The extent to which the assay microorganisms used in this study measured peptides or larger amino acid complexes is not known. However, the PDR indices could not be correlated with their respective biological values if the large polypeptides in the protein-free (tungstic acid filtrates) pepsin digests were hydrolyzed before the digests were analyzed. In this connection, Sheffner, Kirsner and Palmer<sup>5</sup> found that when humans were fed a protein-containing meal, the concentrations of microbiologically non-available polypeptide amino acids of plasma, i.e., large polypeptides, decreased after the meal, whereas concomitantly the microbiologically available plasma amino acids (free amino acids and small polypeptides) were significantly increased. These data suggest, therefore, that if polypeptides are absorbed at an early stage of digestion, they probably consist of relatively few amino acid residues.

<sup>4</sup> Pentex Incorporated, Kankakee, Illinois.

<sup>5</sup> Sheffner, Kirsner and Palmer, unpublished data.

It is apparent that the last step in the calculation of the PDR index predicts that any process which severely decreases peptic digestibility will lower the nutritional value of the treated protein in spite of patterns of subsequently released amino acids which are equally good. The significance of this fact is not yet understood. However, work is continuing to determine the nutritional importance of amino acid patterns during the early stages of absorption, and the effect of peptic digestion upon the subsequent digestion and utilization of protein.

#### SUMMARY

The relationship between the pattern of amino acids released by digestive enzymes and the biological value of food proteins was studied. The pattern of amino acids released *in vitro* by pepsin revealed differences between proteins which were not apparent from their total essential amino acid content nor from the patterns existing when the pepsin digests were further digested with trypsin and crepsin.

An amino acid index is described which takes into account the physiological availability of amino acids during digestion. The new index combines the pattern of essential amino acids released by *in vitro* pepsin digestion with the amino acid pattern of the remainder of the protein to produce an integrated index — the Pepsin-Digest-Residue (PDR) amino acid index. The PDR index was closely correlated with the net protein utilization value of a variety of proteins. Division of the PDR index by the digestibility coefficient of the respective proteins yielded values which accurately predicted the biological values of the proteins studied.

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# INFLUENCE OF FOOD AND ENERGY RESTRICTION AND SUBSEQUENT RECOVERY ON BODY COMPOSITION AND FOOD UTILIZATION OF RATS

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Most animals at some time during their life cycle are likely to undergo periods in which dietary restrictions lower the growth below the maximum obtainable. The influence of diet on life span has been recently reviewed by Silberberg and Silberberg ('55). Osborne and Mendel ('14) first reported that suppression of the growth of rats did not erase the capacity to grow. Clarke and Smith ('38) noted that caloric restriction for three weeks would not prevent rats from attaining the size of the controls. Earlier, Stewart ('16) had also noted that after short periods of starvation rats do have the ability to attain the control size. Factors such as prolonged periods of stunting (Clarke and Smith, '38; Quimby, '48; Jackson and Stewart, '20; and McCay et al., '35, '39) and growth restrictions during suckling (Schultze, '55; and Jackson and Stewart, '20) have not allowed animals to reach full size after a period of liberal feeding. The literature has been reviewed on cattle and interrupted growth by Winchester and Howe ('55) and they have also shown that steers restricted in their energy intake and then refed would attain the size and approximate composition of the controls. The restricted steers made a weight gain equal to that of the controls on about the same amount of feed even though it took a longer period of time.



The influence of nutrient restriction and subsequent recovery on body composition of simple-stomached animals has not previously been studied nor has much attention been given to the total amount of food required due to the interruption of growth and subsequent recovery. Therefore, these experiments were designed to study these factors and to attempt to define experimental conditions more rigidly. This was done by studying both a total food and energy restriction with purified diets and by controlling food intake on the basis of body weight<sup>0.75</sup> (Kleiber, '47).

#### EXPERIMENTAL

The experimental design, ration composition, and food intake in experiment 1 are illustrated in table 1. The rats of lot 1, the control, received the basal ration ad libitum for the entire experimental period. The food intake of the animals in lot 2 was restricted for 28 days, and then they were fed the basal ration ad libitum until they had eaten an amount equal to that consumed by lot 1. Lot 3 was fed in a similar manner to lot 2, except that the rats were restricted in energy intake (sucrose and cottonseed oil) but given an intake of protein, minerals, and vitamins equal to lot 1. The degree of food or energy restriction was 70% of the intake of lot 1, per unit of metabolic body size (body weight<sup>0.75</sup>; Kleiber, '47). Therefore, the actual food intake of lot 2 and the energy received by lot 3 were decreased each week so that each was less than 70% of the amount received by lot 1. These factors, however, remained at 70% on the basis of body weight<sup>0.75</sup>. This was done because of the greater weight increase of the rats in lot 1 and hence a greater maintenance requirement than for lots 2 and 3. The animals of lots 2 and 3 were thus pair-fed with lot 1.

Eighteen male weanling rats (Sprague-Dawley strain) were used in each group. Nine in each group were killed for body composition studies at the end of the restriction period. The remainder were killed at the end of the experiment. Body composition was determined on the body minus the gastro-

intestinal tract and contents. As suggested by Pace and Rathbun ('45) and confirmed by Meyer ('55), body water was determined and the fat-free body calculated from the body water content. The constant of 73.5% water in the fat-free body was used.

The feeding in experiment II was conducted in the same manner as in experiment I except that the food restriction was for 21 days. Weighing conditions were changed slightly. To nullify the influence of the gastrointestinal content on body

TABLE 1  
*Experimental design and food consumption in experiment I*

CONSTITUENTS OF DIET	LOT 1	LOT 2	LOT 3
	Controls	70% food consumption of lot 1 <sup>1</sup>	70% energy consumption of lot 1 <sup>1</sup>
	gm	gm	gm
Average food consumption during restriction period			
Sucrose	173.6	92.2	65.2
Cottonseed oil	12.4	6.6	4.6
Crude casein	49.6	26.4	49.6
Vitamin mix <sup>2,3</sup>	2.5	1.3	2.5
Salts IV <sup>4</sup>	9.9	5.3	9.9
Total	248	131.8	131.8
Average food consumption during recovery period			
Sucrose	170.6	250.7	252.1
Cottonseed oil	12.2	17.9	18.6
Crude casein	48.8	71.5	72.0
Vitamin mix	2.4	3.6	3.6
Salts IV	9.8	14.3	14.4
Total	243.8	357.4	360.1
Grand total	491.8	489.2	491.9

<sup>1</sup> The food consumption of these lots was 70% of the controls per unit of body weight<sup>2</sup>.

<sup>2</sup> The vitamin mix contained the following vitamins in milligrams per 100 gm: thiamine 50, riboflavin 30, pyridoxine 20, niacin 200, calcium pantothenate 200, folic acid 3, biotin 1, and vitamin B<sub>12</sub> 0.2. Sucrose was added to equal 100 gm.

<sup>3</sup> Ample amounts of vitamins A, D, E, and K were supplied weekly by oral administration.

<sup>4</sup> Phillips and Hart, J. Biol. Chem., 100: 657 (1935).

weight, the rats were kept 12 hours without food before weighing at the start and at the end of the experiment. In addition the entire body (including gastrointestinal tract and contents) was analyzed for water. Koch ('56) noted no difference in body composition between rats kept 12 hours without food and those analyzed with the intestinal contents removed.

The analysis for variance for paired data (Snedecor, '40) was the statistical treatment for comparison.

### RESULTS AND DISCUSSION

Table 2 presents the two experiments. These results were similar. Reduction in growth due to either an energy or total food restriction was about equal. An analysis of the carcasses for fat and water and the calculation of fat-free tissue, showed that there were no differences between the two deficiencies. This indicates that the primary deficiency of the total food restriction is one of energy as far as can be measured by gains or body composition.

After realimentation, when the restricted rats were allowed to consume a total amount of food equal to that consumed by the controls, a remarkable ability to recover from this growth interruption was demonstrated. In the first experiment with the 4-week restriction period, both groups of "deficient" rats came within 9 gm of the weight of the ad libitum-fed controls. However, in the second experiment, with a three-week food restriction, there was no statistical difference between the controls and their restricted groups in respect to weight gain. These results are in agreement with those of Clarke and Smith ('38) and Quimby ('48). The work of Winchester and Howell ('55) showed that little extra feed was required by the energy-restricted steers in comparison with the full-fed steers.

The fat and fat-free tissue content of the carcasses or bodies was the same for the rats restricted in energy and those fed ad libitum. This was not true, however, for the rats restricted in total food. These animals, while about the same in weight, had a greater amount of fat and a lesser amount of fat-free tissue. Apparently an advantage is gained for animals during

TABLE 2

*Effect of food and energy restriction and of realimentation upon body composition and food utilization in rats*

	FOOD RESTRICTION			FOOD RESTRICTION PLUS REALIMENTATION		
	Lot 1 Controls	Lot 2 Food re- striction	Lot 3 Energy re- striction	Lot 1 Controls	Lot 2 Food re- striction	Lot 3 Energy re- striction
EXPERIMENT I						
No. of days	28	28	28	42	51	55
Rats per group	18	18	18	9	9	9
Mean gain, gm	114	39 <sup>1</sup>	40 <sup>1</sup>	186.3	177.1 <sup>2</sup>	176.8 <sup>2</sup>
Mean final wt., gm	167	93 <sup>1</sup>	93 <sup>1</sup>	239.1	231	229.7
Carcass data:						
Carcass analyzed	9	9	9			
Mean carcass wt., gm	151.4	86 <sup>1</sup>	83.7 <sup>1</sup>	221.4	213.2	214.7
Fat:						
Wt., gm	16	4.9 <sup>1</sup>	4.9 <sup>1</sup>	25.5	29.4	24.5
Percentage <sup>3</sup>	31	19.1 <sup>1</sup>	19.5 <sup>1</sup>	33.6	38.5 <sup>2</sup>	33.1
Dry fat free body:						
Wt., gm	34.5	20.7 <sup>1</sup>	20.1 <sup>1</sup>	49.9	46.4 <sup>1</sup>	48.5
Percentage <sup>3</sup>	68.4	80.9 <sup>1</sup>	80.5 <sup>1</sup>	66.4	61.5 <sup>2</sup>	66.9
EXPERIMENT II						
No. of days	21	21	21	35	42	41
Rats per group	12	12	12	6	6	6
Mean food consumed, gm	212.3	128.4	128.4	444.8	444.1	442.9
Mean gain, gm	73.2	35.5 <sup>1</sup>	43.3 <sup>1</sup>	149	146.8	140.2
Mean final wt., gm	139.7	104.1 <sup>1</sup>	112.7 <sup>1</sup>	216	215.4	209.6
Composition:						
Rats analyzed	6	6	6			
Fat:						
Wt., gm	11.3	4.3 <sup>1</sup>	4.2 <sup>1</sup>	22.5	24.7	21.8
Percentage <sup>3</sup>	25.4	14.6 <sup>1</sup>	13.1 <sup>1</sup>	30.9	33.6	31.3
Dry fat free body:						
Wt., gm	32.7	25.4 <sup>1</sup>	27.8 <sup>1</sup>	49.5	48.6	47.8
Percentage <sup>3</sup>	74.6	85.4 <sup>1</sup>	86.9 <sup>1</sup>	69.1	69.4	68.7

<sup>1</sup> Highly significant difference from controls.

<sup>2</sup> Significant difference from controls.

<sup>3</sup> Dry basis.

growth interruption if the consumption of protein, vitamins, and minerals is maintained at or above their requirements. This was not apparent at the end of their period of growth restriction but was at the end of the realimentation.

It has been well established that maintenance requirements decrease as undernutrition progresses (Stewart, '16; Thompson and Mendel, '18; Jackson, '37; Forbes et al., '38; and Quimby, '48). This may be due to a lower basal metabolism (Horst et al., '34) or increased digestibility of food (Quimby, '48), or both. Restricted rats in these experiments were able to make gains similar to those of the ad libitum-fed controls when given an equal total food intake even though the total time on experiment was longer. This seems to indicate that the lowered maintenance requirement from restricted feeding was prolonged into the unrestricted feeding period. Winchester and Howe ('55) suggested this for steers. Also it has been reported by Horst et al. ('34) that lowered basal metabolism induced by starvation continued about 7 days after adequate nutrition was initiated.

#### SUMMARY

Growing rats were restricted in their food or energy intake for three or 4 weeks at 70% of the food intake per unit of body weight<sup>0.75</sup> of the ad libitum-fed controls. They were subsequently fed a total amount of food equivalent to that consumed by the controls. The rats in the first experiment in which the food and energy intake were restricted for 4 weeks came within 9 gm of the weight gain of the controls. This was accomplished even though the experimental period was longer for the restricted rats. In the second experiment there was statistically no difference between groups in weight gains.

Even though there was little difference in weight gains between rats restricted either by energy or total food intake, differences were apparent in body composition. The proportion of fat to fat-free tissue for the rats restricted in energy intake and subsequently refed was the same as that of the

ad libitum-fed controls. However, a greater proportion of fat was found in the bodies of the rats which were restricted in total food intake and subsequently fed adequately.

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# EFFECT OF ADDED LYSINE ON GROWTH OF RATS FED A CEREAL AND MILK DIET

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(Received for publication April 12, 1956)

## INTRODUCTION

Studies by Rosenberg and co-workers ('52, '54) have shown that the addition of L-lysine to a bread diet supplemented with fat, salts and vitamins, increases the rate of growth of weanling rats. The bread was made with wheat flour and contained three parts of nonfat milk solids and two parts of yeast per 100 parts of flour. A deficiency of lysine was to be expected in a diet composed mainly of wheat flour. Many workers in nutrition since Osborne and Mendel ('14) have shown that the proteins of wheat and other grains are low in lysine. Since these grains are also used in the manufacture of cereals, which are almost invariably eaten with milk, a study was set up to determine whether the addition of L-lysine to a cereal and milk diet would enhance its nutritive value. E. B. Hart ('52) pointed out the shortcomings of cereal proteins but since they are served with milk, he recommended that the protein value of the combination should be considered since the milk would dissipate any inferiority of the cereal in respect to its protein.

In the present experiments the effect of addition of L-lysine to a diet of cereal<sup>1</sup> and powdered whole milk on the growth of male weanling rats was determined. One ounce of this cereal is usually mixed with 5 oz. of fluid milk in infant feed-

<sup>1</sup>The cereal used in these diets was Pabulum Mixed Cereal (Mead Johnson and Co.), consisting of wheat meal (farina), oatmeal, yellow corn meal, wheat germ, tribasic calcium phosphate, powdered alfalfa leaf, dried yeast, sodium chloride and reduced iron.



ing. In the present experiment the basal diet contained 70% of cereal and 30% of powdered milk, equivalent to a mixture of 1 oz. of cereal and 3.5 oz. of fluid milk.

#### EXPERIMENTAL

Six groups of 10 male weanling rats each were housed in individual screen-bottom cages, maintained in an air-conditioned room at 76 to 78° F. and fed the experimental diets for 6 weeks. The basal diet, 1, consisted of 70% cereal and 30% powdered whole milk. Diets 2 and 3 contained in addition, 0.14 and 0.28% of L-lysine,<sup>2</sup> respectively. This is equivalent to adding 0.2 and 0.4% of L-lysine to the cereal component of the diet. These levels of lysine added to a bread diet have been shown to give marked stimulation of growth (Rosenberg et al., '52, '54). Diets 4, 5 and 6 corresponded to diets 1, 2 and 3, respectively, and contained in addition a vitamin mixture containing B vitamins, ascorbic acid and fat soluble vitamins (Sarett and Snipper, '54) to make sure that the growth response was not limited by any vitamin inadequacy. Calculations showed that all of the diets were adequate in regard to the minerals required for growth of rats. The animals were weighed each week and records were kept of food and water intake during the course of the experiment.

#### RESULTS

The average weight gain and food and water intake for each group of animals are shown in table 1. On diet 1, consisting of only cereal and powdered milk, the animals gained an average of 192 gm in 6 weeks, whereas on the 5 diets supplemented with L-lysine or vitamins or both, the average gains were 200 to 204 gm. There was no significant difference between the weight gain on diet 1 and that found on any of the other diets. A comparison of weight gain on diets 1 and 2 showed a non-significant p value of approximately 0.2.

<sup>2</sup> L-Lysine was added in the form of Darvyl (du Pont Co.) containing 95% of L-lysine · HCl and 5% of D-lysine · HCl.

Diet 4 consisted of cereal and milk supplemented with vitamins and supported an average weight gain of 202 gm. With L-lysine also added, diets 5 and 6, weight gains of 200 and 204 gm, respectively, were obtained. These data show that the growth of weanling rats on a cereal and milk diet was not significantly improved by the addition of L-lysine or a vitamin

TABLE 1

*Effect of added L-lysine and vitamins on growth of male weanling rats on a diet of 70% cereal and 30% powdered whole milk*

	DIET 1	DIET 2	DIET 3	DIET 4	DIET 5	DIET 6
Added vitamins <sup>1</sup>	—	—	—	+	+	+
Added L-lysine, %	..	0.14	0.28	..	0.14	0.28
No. of rats	10	10	9	8	9	10
Wt. gain, gm per rat						
1 wk.	29	28	29	27	30	32
2 wk.	61	63	62	63	61	66
3 wk.	96	99	99	100	96	101
4 wk.	126	134	128	132	131	133
5 wk.	163	174	164	170	170	173
6 wk. <sup>2</sup>	192 ± 20	204 ± 20	203 ± 17	202 ± 15	200 ± 18	204 ± 38
Food intake, gm per 6 wk. <sup>2</sup>	501 ± 42	530 ± 30	520 ± 39	532 ± 26	527 ± 50	564 ± 89
Food efficiency, gm gain per 100 gm food <sup>2</sup>	38 ± 2	39 ± 3	39 ± 2	38 ± 2	38 ± 2	37 ± 6
Water intake, ml per 6 wk.	1030	1070	990	930	910	1010
ml per gm food <sup>2</sup>	2.1 ± 0.3	2.0 ± 0.3	1.9 ± 0.4	1.7 ± 0.2	1.7 ± 0.3	1.8 ± 0.4

<sup>1</sup> Sarett and Snipper ('54).

<sup>2</sup> Values given with standard deviations.

mixture or both. Growth on these diets was about the same as that obtained with good stock or experimental diets.

The average food intake on each of the 6 diets varied from 501 to 564 gm for the 6-week period, and the food efficiencies were calculated to be 37 to 39 gm gained per 100 gm of food. There were no significant differences in average food intake or food efficiency values among the groups on the six diets. The average water intakes were 912 to 1070 ml and ranged from 1.7 to 2.1 ml of water per gram of food. The differences between groups were not significant.

#### DISCUSSION

Feeding of a diet containing 70% cereal and 30% dried milk solids (a proportion containing less milk than would be used in feeding the cereal to infants) results in good growth of rats which is not significantly improved by the addition of a lysine or vitamin supplement. Milk and cereal complement each other; e.g., the low levels of riboflavin and certain amino acids in cereals are made up by the milk, and the deficiency of iron in milk is overcome by the cereal.

In the above diet about 60% of the protein was supplied by the cereal (16% protein) and 40% by the powdered milk (25% protein). This level of milk protein makes up for the amino acid deficiency in the proteins of most cereal products. This point has been emphasized by Hart ('52) in discussing the utilization of cereal proteins.

The level of lysine in the bread diet of Rosenberg et al. ('52, '54) was 0.3 to 0.4%, whereas Rose ('37) has shown that 1% of lysine is needed in the diet for good growth of rats when a complete amino acid mixture is provided. In the present study, the cereal contained 0.69% of lysine (microbiological assay) and powdered whole milk has been calculated to contain about 2% of lysine (Macy et al., '53). The lysine content of diet 1 can therefore be calculated as follows: from 70 gm of cereal, 483 mg of lysine; from 30 gm of powdered whole milk, 600 mg of lysine; total, 1083 mg of lysine/100 gm diet.

This mixture of cereal and milk contains 1.08% of lysine, which exceeds Rose's requirements for rat growth. If 39% milk solids were used in the diet (as would be obtained from a mixture of 1 oz. of cereal and 5 oz. of fluid milk), the milk alone would provide 780 mg of lysine per 100 gm diet (on a dry weight basis), and only a small amount of lysine in the cereal would be needed to bring the lysine value up to 1%.

From the data on food intake the average lysine intakes of the rats on diets 1 and 4 have been calculated for each week

TABLE 2  
*Change in lysine intake during growth of rats*

		WEEKS AFTER WEANING					
		1	2	3	4	5	6
Diet 1	Rat weight, <sup>1</sup> gm	64.7	95.0	128.5	161.1	194.7	228.7
	Lysine intake, mg/day	82	111	131	137	147	164
	Lysine intake, mg/kg/day	1265	1170	1010	850	755	715
Diet 4	Rat weight, <sup>1</sup> gm	63.2	94.4	130.8	165.6	200.1	235.1
	Lysine intake, mg/day	81	117	140	150	162	173
	Lysine intake, mg/kg/day	1280	1245	1065	905	810	735

<sup>1</sup> Average midweek weights.

of the experiment. The lysine intakes per kilogram of body weight were based on the midweek weights of the rats (table 2). The data show that the lysine intake per kilogram of body weight decreased each week as the rats grew. During the first week following weaning less than 1300 mg of lysine per kilogram per day supported good growth. This is approximately half the figure of 2750 mg reported as required for growth of the 50 gm rat by Albritton ('53).

#### SUMMARY

The gain in weight of male weanling rats on a diet of 70% cereal and 30% powdered whole milk was not significantly

increased by the addition to the diet of 0.14 or 0.28% of L-lysine (equivalent to 0.2 or 0.4% of L-lysine added to the cereal). Addition of a supplementary vitamin mixture had no significant effect on weight gain with or without added lysine in the diet.

The diet of 70% cereal and 30% powdered milk contained approximately 1.08% lysine, which exceeds the requirement for growth, 1%, found by Rose ('37). A mixture of 61% cereal and 39% powdered milk (which is equivalent to one part cereal and 5 parts fluid milk such as used in infant feeding) contains approximately 1.2% lysine.

On the diet of 70% cereal and 30% powdered milk rats grew as well as they do on stock diets or on good experimental diets used in these laboratories. From the data on food intake during the growth period it has been calculated that the daily lysine intake decreased from about 1275 mg per kilogram of body weight during the first week after weaning (average midweek weight, 65 gm) to about 725 mg per kilogram during the 6th week (average midweek weight, 230 gm).

#### ACKNOWLEDGMENTS

The author wishes to thank Dr. Lawrence P. Snipper for handling some of the details of this experiment, and Wilbur Martin and John Kissel for care of the animals.

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# ALKALINE PHOSPHATASE ACTIVITY AND DIET

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Significantly higher than normal values for alkaline phosphatase activity had been found for the liver of adult rats that had been maintained on a protein-deficient — high carbohydrate diet (Ross, Ely and Archer, '53; Ross and Ely, '54). The question arose whether the amount of protein included in the diet was directly correlated with the amount of hepatic alkaline phosphatase activity. Plasma and intestinal alkaline phosphatase activity was shown to be affected by the quantity (Lawrie and Yudkin, '49) or quality (Weil and Russell, '40) of lipid material in the diet. The activity of hepatic alkaline phosphatase in rats kept on specially planned rations has been found to be controlled by the absolute amount of casein and dextrose and by the proportion of casein and dextrose in the diet.

## PROCEDURE

Male rats of the Wistar strain weighing 175 to 190 gm were used, 696 in all. After weaning, these rats had been maintained on a commercial food which contains approximately 23% protein.<sup>1</sup> The rats were then maintained for 21 to 23 days on semi-synthetic diets. These diets were designed with varying proportions of washed casein as the protein and of dextrose as the carbohydrate. The content of fat, salts and vitamins was held constant. All diets contained salt mixture USP XII, 4%; corn oil, 5%; rice bran extract.<sup>2</sup> 2%, supplemented with

<sup>1</sup> Purina Fox Food Blox.

<sup>2</sup> Vitab.



0.002% riboflavin. Non-nutritive fiber (NNF) was used as a filler. The rats had access to water at all times.

At the conclusion of the test feeding period, rats from each group were decapitated. Weighed pieces of liver were immediately homogenized in cold water for 30 seconds in a Waring Blendor at low speed. Sodium glycerophosphate, 5 mg/ml, at pH 9.1, was used as the substrate for the alkaline phosphatase determinations which were made at 37°C. on aliquot portions of this liver homogenate (Ross, Ely and Archer, '53). The unit of activity used was the micrograms of phosphorus liberated per milligram of fresh liver per hour.

#### RESULTS AND DISCUSSION

##### *Different casein and dextrose proportions in the diet*

In order to determine the effect on alkaline phosphatase activity of different casein and dextrose proportions, 9 groups of 12 rats each were maintained on 9 diets, in which the casein plus dextrose was always 80% of the total food mixture. Each diet contained 2% vitamin, 5% oil, 4% minerals and 9% non-nutritive fiber. The voluntary food intake of the rats was determined daily during the feeding period.

Alkaline phosphatase activity was found to be related (1) inversely to the amount of casein in the diet up to 30% (fig. 1); (2) beyond 40%, directly to the amount of casein in the diet; (3) directly to the amount of dextrose in the diet down to 50%; and (4) inversely to the dextrose content of the diet below 40%. Minimum enzyme values were therefore found when diets contained 30 to 40% of casein and 50 to 40% of dextrose.

The high alkaline phosphatase activity per unit weight of fresh liver found when rats were maintained on a casein-free or dextrose-free diet may represent an increase (a) in the amount of total enzyme in a given amount of tissue, (b) in amount of active enzyme, or (c) in the concentration of enzyme substance within the cell. It had been shown by Ely and Ross, ('53) that the level of hepatic alkaline phosphatase activity of

rats maintained on a protein-free diet for a considerable period of time was unchanged for the individual cell. On the other hand, the enzyme level had been shown (Ely and Ross, '54) to change more rapidly than the number of cells per unit mass of fresh tissue indicating that the increase in activity is the result of both changes in concentration and in amount.

Voluntary food intake was lowest in those rats maintained on either a casein-free or dextrose-free diet (table 1). Weight

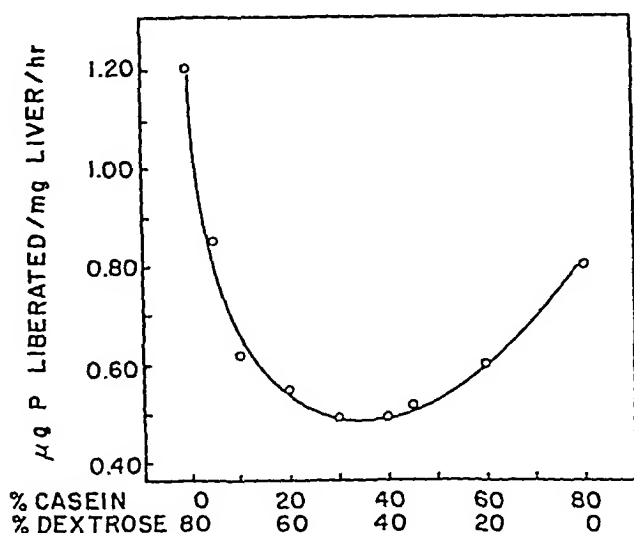


Fig. 1 Effect on hepatic alkaline phosphatase activity of diets containing different proportions of casein and dextrose.

TABLE 1

*Food intake and body weight of rats maintained on diets differing in proportions of casein and dextrose*

CASEIN	DEXTROSE	AVERAGE FOOD INTAKE DURING FEEDING PERIOD	AVERAGE BAT WEIGHT	
			Initial	Final
%	%	gm	gm	gm
0	80	11.3	186	134
10	70	15.4	186	221
20	60	14.8	185	220
30	50	17.3	185	246
45	35	15.1	186	244
60	20	12.5	186	224
80	0	12.6	186	207

loss was apparent in rats on the casein-free diet and weight gain was greatest in those receiving the 30 to 45% casein and 50 to 35% dextrose diets respectively.

### *Different amounts of a diet*

The effect of different amounts of a diet on alkaline phosphatase activity was therefore tested. Three groups of 20 rats each were maintained on three different amounts of a diet containing 20% casein and 36% dextrose. The oil, minerals and vitamins were added to the diet in such amounts

TABLE 2  
*Alkaline phosphatase value and amount of food intake*

	DIETARY INGREDIENTS					N.N.F.	FOOD IN- TAKE	ALKALINE PHOSPHATASE ACTIVITY
	Casein	Dex- trose	Oil	Min- erals	Vita- mins			
							gm	units
Group I								
% of diet	20	36	5	4	2	33		0.741 ± 0.057 <sup>1</sup>
gm/day	3.2	5.8	0.8	0.64	0.32	5.3	16	
Group II								
% of diet	20	36	10	8	4	22		
gm/day	1.6	2.9	0.8	0.64	0.32	1.8	8	0.699 ± 0.047
Group III								
% of diet	20	36	20	16	8	0		
gm/day	0.8	1.4	0.8	0.64	0.32	0	4	0.657 ± 0.047

<sup>1</sup> Standard error of the mean.

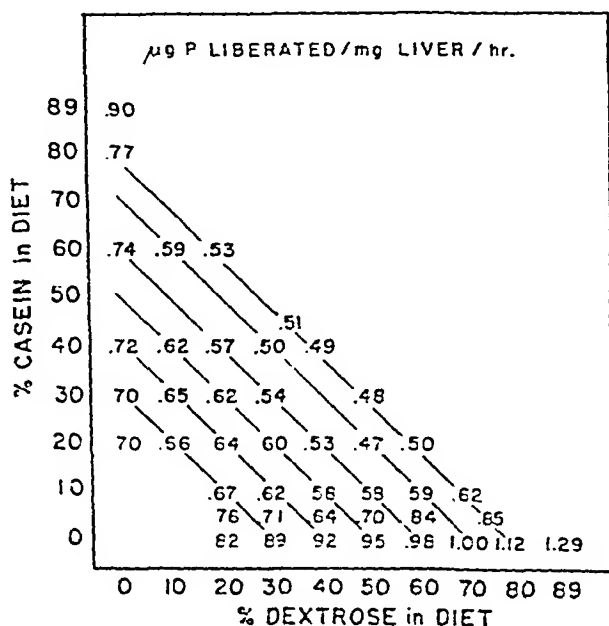
that every rat consumed identical quantities of these ingredients (table 2). Rats of group I consumed on the average 16 gm of food per day. Rats of group II were given one-half this ration or 8 gm per day, and group III rats one-quarter the ration or 4 gm per day.

A slight decrease in alkaline phosphatase activity was found with decreasing food consumption; however, the activity was not found to vary significantly or appreciably with the amount of food consumed (table 2). Since the lowest activity was

found for those rats which consumed the largest amount of food and the highest activity for those which consumed the smallest amount when different proportions of casein and dextrose were consumed ad libitum, the amount of food consumed would not seem to be the determining factor for the amount of alkaline phosphatase activity.

*Different amounts of casein and of dextrose*

In order to determine the effect on alkaline phosphatase of different amounts of casein and of dextrose, 43 groups of 12 rats each were maintained on 43 diets in which the amount of casein was varied for each level of dextrose and vice versa. Non-nutritive fiber was added to the diets in amounts necessary to make the sum of the casein, dextrose and non-nutritive fiber 89%. Each diet contained 2% vitamins, 5% oil and 4% minerals. The data presented in figure 2 show the effect on



alkaline phosphatase activity of diets of the same casein content but differing in dextrose content (vertical columns), the effect of diets of the same dextrose content but differing in casein content (horizontal lines), and the effect of diets differing in both dextrose and casein content but similar in caloric content (diagonals). Standard errors have been omitted in figure 2 because the added figures would make comparisons more difficult. The differences found are statistically significant.

*Variation in the level of casein or of dextrose.* For casein alone, or for dextrose alone, hepatic alkaline phosphatase activity was found to rise with concentration of the constituent in the diet (fig. 2). The rise with concentration was more striking for dextrose than for casein. Addition of small amounts of casein to a dextrose diet, or of small amounts of dextrose to a casein diet, reduced the degree of the change in activity; sufficiently large amounts tended to reverse the effect so that the activity fell with increased concentration of the component in the diet. Dextrose, however, was a more potent factor than casein in producing these changes. For example, 5% casein reversed the effect for dextrose only when the dextrose concentration was 30% or less. Similarly, 10% casein reversed the effect for dextrose only at dextrose concentrations of 40% or less, while 10% dextrose reversed the effect for casein at casein concentrations as great as 60%. The lowest values for alkaline phosphatase activity therefore were found in rats fed diets containing approximately equal proportions of casein and dextrose.

*Variation in the level of both casein and dextrose.* A comparison was made of the effects of variation in the amounts of casein and dextrose where the sum of the casein plus dextrose was essentially constant (diagonal lines in figure 2). Since the casein used gave a nitrogen analysis of 13.8% (86% crude protein) the diets which had the same sum of casein plus dextrose were essentially constant in caloric value. Alkaline phosphatase activities for each of these groups of diets varied with the proportion of casein to dextrose. From group to

group, the activities also varied with the caloric content of the diet. The highest activity in each group of calorically similar diets was found to be directly related to the caloric content of the diet (fig. 3). On the other hand, the lowest activity in each group of these diets was found to be inversely related to the caloric content of the diet. When the caloric content was lower the amount of change was less, whether maximum or minimum.

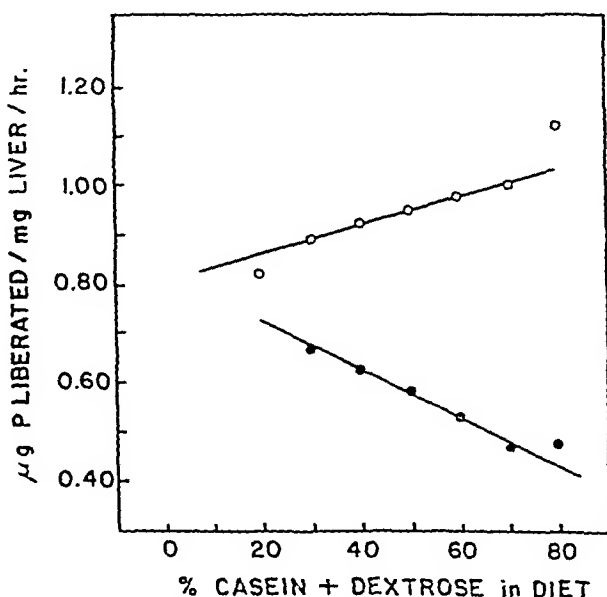


Fig. 3 Effect on alkaline phosphatase activity of groups of diets calorically similar. ○ = maximum activity in a group of calorically similar diets. ● = minimum activity in a group of calorically similar diets.

#### SUMMARY

Alkaline phosphatase activity of rat liver has been found to be sensitive to variation in the proportion of casein and dextrose in the diet. Activity values were found to be high not only when there was a large proportion of dextrose but also when there was a large proportion of casein in the diet. Low activity values were found when these dietary constituents were present in approximately equal proportions. Alkaline

phosphatase activity was found to be directly affected by variation in the casein content when the dextrose content was held constant and by variation in the dextrose content when the casein content was held constant. A variation in the amount or in the proportion of the dietary factors, casein and dextrose, has been shown to alter the alkaline phosphatase activity.

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PROCEEDINGS  
OF THE TWENTIETH ANNUAL MEETING  
OF THE AMERICAN INSTITUTE  
OF NUTRITION

CONVENTION HALL, ATLANTIC CITY, NEW JERSEY

APRIL 15-20, 1956

COUNCIL MEETINGS

Council meetings were held at Chalfonte-Haddon Hall, Atlantic City, on Sunday, April 15, and Monday, April 16. Formal actions of the Council are reported in the following minutes of the two business meetings held on April 17 and April 19 in Convention Hall.

SCIENTIFIC SESSIONS

One hundred and thirty-two papers were submitted by members. Ten of these were read by title, 5 were transferred to biological chemistry, one to physiology, and 12 to inter-society sessions. Thus 104 papers submitted by members, together with 18 received from other member societies (14 from biological chemistry, two from pathology, one from physiology, and one from immunology), were arranged into 12 half-day sessions. In addition, one half-day session was devoted to a symposium entitled "Amino Acids and Proteins."

BUSINESS MEETINGS

1. *Minutes.* The minutes of the 19th annual meeting, as published in the September 1955 issue of the *Journal of Nutrition*, were approved.



2. *Election.* The secretary transmitted the ballots to the Tellers' Committee, Dr. B. S. Schweigert and Dr. A. Arnold. The committee reported election results on 227 ballots cast as follows:

President: H. J. Deuel, Jr.

Vice President: R. R. Williams

Treasurer: John B. Brown (3-year term)

Councillor: E. W. McHenry

Associate Editors (4-year term beginning May 1, 1956):

Clarence P. Berg

C. G. Mackenzie

H. R. Bird

On the basis of the suggestions on the ballot returns, President Sebrell appointed the following Nominating Committee for 1956-1957:

D. V. Frost, Chairman

W. J. Darby

D. M. Hegsted

J. B. Allison

C. A. Baumann

3. *Membership.* The Secretary reported that as of April 1, 1956, there were 388 active members and 41 retired members, or a total of 429 members in the American Institute of Nutrition. The following members passed away during the year:

Dr. C. Robert Moulton, April, 1955

Dr. E. G. Ritzman, May 15, 1955

Dr. Henry C. Sherman, October 8, 1955 (Past President)

Dr. Kate Daum, December 31, 1955

The following resolution was adopted with respect to the late past president Sherman:

"The American Institute of Nutrition has learned, with regret, of the passing away on October 8, 1955, of Professor Henry Clapp Sherman, one of the prime organizers and an early president of the Institute. The members of the Institute here assembled in the twentieth annual meeting do hereby call attention to the many valuable contributions to nutritional science which were made by Professor Sherman, and to the significance of his important part in the establish-

ment, first of the *Journal of Nutrition*, and later of this Institute itself.

"The Institute wishes to pay tribute to his memory by placing this resolution in the Minutes of the Society, and by communicating these sentiments of respect and condolence to the bereaved family.

"It is especially fitting that a copy of this resolution be sent to the late Professor Sherman's daughter — Caroline Sherman Lanford — an authority on nutrition in her own right."

4. *Treasury.* The Auditing Committee, G. M. Briggs and A. E. Schaefer, submitted a written report that the report of the Treasurer was substantiated by the records of the Treasurer's Office. The Treasurer's and Auditors' reports were approved, and dues were approved at \$1.00 per member for the coming year. By voice vote the outgoing Treasurer, O. L. Kline, was commended for the efficient and prompt manner in which his office functioned during his three-year term.

5. *Journal of Nutrition.* The Journal Editor, G. R. Cowgill, submitted an annual report, the summary of which follows:

Volumes of the <i>Journal of Nutrition</i> , 1955	Nos. 55, 56, 57
Number of papers submitted	188
Number of papers rejected	23
Number of papers published	150
Number of pages per article	12.3
Number of biographies	3

The papers published during the year covered research carried on in 89 laboratories representing 74 institutions. Due to the soundness and financial success of the *Journal* as reported by The Wistar Institute, its publisher, the cost of the three volumes of the *Journal* to members for the year 1956-57 has been temporarily reduced from \$7.50 to \$6.50. In addition, Wistar Institute has agreed to print all stationery, bills, and other printed matter required by our Institute in its normal operation at no charge.

The Editorial Board adopted in principle the policy that the *Journal* does not look with favor upon the wide use of *Federation Proceedings* abstract issue as a journal to be used in "Literature Cited." It was felt that references to abstracts in general would not be acceptable unless unusual circumstances merited their use.

The policy of publishing supplements to regular issues of the *Journal* will be continued if authors agree to the costs involved.

The Editor's report was approved with a vote of appreciation to Dr. Cowgill for the efficient handling of *Journal* affairs.

#### 6. *Reports of Standing Committees:*

- (a) Joint Committee on Nomenclature (C. K. King and E. M. Nelson). The Committee recommended that the trivial name for 1,2-dithiolane-3-valeric acid be "lipoic acid." The Committee also recommended disapproval of the recommendation of the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry that the term pyridoxine be used as a group name of naturally-occurring pyridine derivations with vitamin B<sub>6</sub> activity. It was felt that "vitamin B<sub>6</sub>" is a more suitable group name. The resignation of E. M. Nelson from the Committee, effective June 30, 1956, was a part of the report. The report was approved with a vote of thanks to Dr. Nelson for his long and faithful service on the Committee.
- (b) The Committee on the Registry of Pathology of Nutritional Diseases (H. Pollack) reported that reorganization and coordination of effort with a similar committee operating under the American Society of Experimental Pathology has consumed the efforts of the group during the past year.

- (c) Representative to the Food and Nutrition Board and the Division of Biology and Agriculture, N.R.C. (N. R. Ellis). The report included a recommendation that our Institute consider favorably the matter of extending financial assistance to defray the costs of distributing to high schools and junior colleges the N.R.C. publication, "The Challenge of the Life Sciences." Upon the recommendation of the Council, the report was approved and the Treasurer was authorized to pay out of the Institute Treasury a sum of \$50.00 payable to Paul Weiss, Chairman, the Biology Council, N.R.C., to aid in the distribution of the above named publication.
- (d) Representatives on the AAAS Council (J. H. Roe and P. B. Pearson) reported on the AAAS Council meetings held in Atlanta, Georgia, in December, 1955. The report contained the recommendations that our Institute should take advantage of additional programing of nutrition studies by extending sessions in this area at AAAS scientific meetings. The AAAS Council felt it desirable to secure opinions from affiliated societies regarding the desirability of assessing affiliated member societies modest sums (\$25.00) that might be used to help defray travel expenses of delegates to Council meetings. Upon the recommendation of our representative on the AAAS Council (Dr. J. H. Roe), this proposal was not approved. The overall report was approved as presented.
- (e) Representative to the Nutrition Division of F.A.O. (P. E. Howe). In the absence of Dr. Howe, Dr. J. H. Roe served as representative for our Institute at the October, 1955, meeting of the National Conference on F.A.O. Doctor Roe's report, which included the recommendation that at future National F.A.O. meetings conference on problems of nutrition education should receive consideration, was approved as presented.

7. *Nominations for Membership.* The Council received 24 nominations for membership. The following 16 were recommended by the Council and approved:

L. A. Bavetta	Olaf Mickelsen
D. E. Becker	O. Neal Miller
Louise J. Daniels	Lura Mae Odland
Burt W. Heywang	Robert Van Reen
Edward G. High	Harold M. Scott
Theodore B. Van Itallie	Martha Trulson
Herman F. Kraybill	Walter G. Unglaub
R. J. Lillie	C. M. Young

8. *Actions of the Federation Board.* The Secretary reported on the actions of the Federation Board in its Sunday meeting as follows:

- (a) The proposal that an Executive Committee be set up within the membership of the Federation Board was defeated.
- (b) The Public Information Committee of the Federation Board (I. M. Hoobler is American Institute of Nutrition representative on the committee) recommended the organization of a year-round Public Information service at Federation Headquarters. The proposal was approved by the Board in principle, and the committee was authorized to seek funds for the expenses involved in committee meetings that would need to be held during the coming year in order to formulate a definite plan for effecting a year-round public information service at Federation Headquarters.
- (c) The proposal that in the future, abstracts of "Papers Read by Title" no longer be printed in *Federation Proceedings* was approved.
- (d) The Board voted the Federation assessment of \$4.00 per member, tentatively adopted in 1955, to be continued, with the acknowledgment that the \$4.00 assessment is entirely used to pay costs of publishing *Federation Proceedings*.

The report was approved as presented with the recommendation that our society also consider deletion of

“Read by Title” papers from the program issue of *Federation Proceedings*.

9. *Plans for International Nutrition Congress in the United States in 1960.* C. G. King, in the absence of Chairman Paul György, reported briefly on the actions of the committee authorized last year to effect preliminary planning for the 1960 International Nutrition Congress in the United States. Pertinent action was as follows: Approval of the committee report that C. G. King serve as President of the 1960 International Nutrition delegate to the 1957 International Nutrition Congress in Paris, France. W. H. Sebrell, Jr., was designated as alternate delegate to the 1957 Congress.

The committee report was approved as presented.

10. *Miscellaneous items.*

- (a) Increasing the honorarium to the Secretary's Office for secretarial assistance to \$150.00 annually was approved. An honorarium of \$50.00 for secretarial assistance for the Treasurer's Office was approved.
- (b) The following resolution was prepared for these minutes:

RESOLUTION

Be it resolved that the American Institute of Nutrition, assembled in Atlantic City, New Jersey, in its annual meeting, April 19, 1956, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of its President-Elect, Harry J. Deuel, Jr., and

Be it further resolved that high tribute be paid to President-Elect Deuel for his outstanding scientific accomplishments in research in nutritional science, for his definitive record in the preparation of scientific publications, for his extraordinary record as an ambassador in furthering international cooperation among scientists, and for his superb contribution to the fellowship of this Society, and

Be it further resolved that the Secretary of the Society be directed to send a copy of this resolution to his wife, Mrs. Grace Deuel, and to his institution, the University of Southern California.

In view of the late Dr. Deuel's many contributions in nutrition research and his devotion to American In-

stitute of Nutrition affairs, the membership adopted the recommendation of the Council that the official record will indicate the election of Dr. H. J. Deuel, Jr., as President for 1956-57 and that R. R. Williams, Vice-President Elect, be delegated to serve as Acting President for the year 1956-57.

- (c) A letter commending the American Society of Biological Chemists for their Fiftieth Anniversary Celebration was forwarded by the Council.
- (d) The procedure of providing an inner envelope for the ballot to be mailed inside the signed envelope, as a modified election procedure, was approved.

#### ANNUAL DINNER AND PRESENTATION OF AWARDS

The annual dinner of the American Institute of Nutrition was held on Wednesday, April 18, in the Madison Hotel, and was attended by 256 members and guests. The program highlight was the presentation of awards by President Sebrell.

The Borden Award in Nutrition was presented to Dr. Frank M. Strong of the University of Wisconsin "for outstanding contributions on the nutritive significance of the components of milk." Doctor Strong's achievements as a research worker were described by Dr. C. A. Elvehjem. Doctor Strong responded by extending his deep appreciation to the Borden Foundation for providing the Award, to nutrition co-workers for honoring him with the Award, and to his many co-workers that have aided in his professional advancement.

The Osborne and Mendel Award was presented to Dr. A. G. Hogan, Professor Emeritus at the University of Missouri, "for his development of synthetic rations for use in nutritional studies and for his original investigations in the field of biochemistry and nutrition, which have made him one of the greatest contributors to the development of our present knowledge of animal nutrition." Doctor Hogan's achievements in nutrition were described by Dr. A. H. Smith. Doctor Hogan responded with some recollections of his early experiences with Dr. Osborne and Dr. Mendel as a graduate student.

COMMITTEES FOR 1956-57

The following are the standing committees beginning July 1, 1956:

*Committee on Registry of Pathology of Nutritional Diseases*

Herbert Pollack, Chairman	W. H. Sebrell, Jr.
O. A. Bessey	C. L. Pirani, Secretary
Consultant, Paul Klemperer	

*Representatives to the Joint Committee on Nomenclature*

C. G. King  
O. L. Kline

*Representative to the Division of Biology and Agriculture, to the Agricultural Research Institute and to the Food and Nutrition Board, National Research Council*

N. R. Ellis

*Representatives to the American Association for the Advancement of Science*

Howard A. Schneider, Section C (Chemistry)  
Paul B. Pearson, Section N (Medical Science)

*Representative to FAO*

Paul E. Howe

*Representative on Federation Public Information Committee*

Lele Macy Hoobler

Respectfully submitted,

R. W. ENGEL, Secretary  
American Institute of Nutrition





## OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award.

*Chairman, Nominating Committee:*

**R. V. BOUCHER**

*Agricultural and Biological Chemistry  
Pennsylvania State University  
University Park, Pennsylvania*

## BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1957 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this honor.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five* copies of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award

*Chairman, Nominating Committee:*

C. G. MACKENZIE

*Department of Biochemistry*

*University of Colorado School of Medicine*

*4200 E. 9th Avenue, Denver 7, Colorado*

# EFFECTS OF DIETARY SUPPLEMENTS IN PREVENTING OR AUGMENTING THE PRODUCTION OF CATARACTS IN RATS BY 1,4-DIMETHANE- SULFONOXYBUTANE<sup>1</sup>

AMOS E. LIGHT, CYRIL SOLOMON AND E. J. DE BEER  
*Wellcome Research Laboratories, Tuckahoe, and The French Hospital,  
New York, New York*

(Received for publication January 3, 1956)

It has been reported by Solomon, Light and de Beer ('55) that busulfan, 1,4-dimethanesulfonxybutane,<sup>2</sup> will produce cataracts when fed to growing rats. As reviewed by Shlaifer ('54), it is well known that the lens structure is susceptible to nutritional disturbances generated by certain vitamin deficiencies or high dietary levels of a particular monosaccharide, galactose. It was therefore decided to investigate the possibilities of altering the course of the 1,4-dimethanesulfonxybutane cataract by modifying the diet through the addition of various substances capable of altering metabolic processes.

Busulfan was synthesized by Timmis ('50) during a search for anticarcinogenic chemicals (Haddow and Timmis, '53). It was found to inhibit the growth of Walker rat carcinoma no. 256 and to depress the number of circulating neutrophils. Clinical trials have shown that this compound is useful in the treatment of chronic myelocytic leukemia in humans (Galton, '53; Galton and Till, '53; Wintrobe et al., '54; Petrakis et

<sup>1</sup> Presented in part at the 20th annual meeting of the American Institute of Nutrition at Atlantic City, N. J., April, 1956. See *Proceedings of the Federation of American Societies for Experimental Biology*, 15: no. 1, Part II, p. 738, 1956.

<sup>2</sup> Supplied as "Myleran" Brand Busulfan (1,4-Dimethanesulfonxybutane), Burroughs Wellcome & Co., (U.S.A.), Inc. Tuckahoe 7, N. Y.

al., '54). These and other effects are similar to those following radiation treatment and 1,4-dimethanesulfonoxybutane has therefore been classed as a radiomimetic drug (Elson, '55; Bergel, '55). However, no cataracts attributable to the drug have been discovered in any other species including humans up to the present time.

This report describes the production of cataracts which appeared in rats during prolonged toxicity tests of busulfan using dose levels over 5 times the recommended therapeutic amount. The prevention and augmentation of these opacities with dietary and other supplements are likewise discussed. Since the cause of cataract formation is still an enigma, any pertinent information may prove useful in the study of this condition.

#### EXPERIMENTAL

*a. Toxicity and cataract production.* Recently weaned male albino rats<sup>3</sup> were fed diets of ground Fox Chow with meat meal<sup>4</sup> containing amounts of busulfan ranging from 2.5 to 1,000 mg of drug per kilogram of diet. For the lower dosage levels a stock diet containing 100 mg per kilogram was first prepared. This was then diluted further to obtain the desired final concentration of the drug. Ten rats were used on each dose level. Hematological studies and autopsies were conducted, where possible, at the end of the experimental periods of 84 and 182 days. Blood sugar values were determined on the rats receiving the dose of 10 mg per kilogram of diet. Although an ophthalmoscope was used at first to detect opacities it was soon found that the cataracts were readily discernible without aid and so were recorded as they appeared grossly.

*b. Non-reversibility of busulfan cataracts.* In order to determine whether or not the cataracts were easily reversible, rats receiving doses of 15 and 20 mg of busulfan per kilogram of diet were transferred to the drug-free control diet as soon

<sup>3</sup> Carworth Farms.

<sup>4</sup> Purina.

as lens opacities were observed. Surviving animals were observed on this drug-free regime for an additional 12 weeks.

*C. Prevention and augmentation of cataract formation with dietary and other biological supplements.* Various substances were tested for their ability to delay death or cataract formation due to busulfan at dose levels of 10, 15 and 20 mg per kilogram of diet. Five or 10 rats were used in each group and the supplements were incorporated in the diet or injected as indicated. Likewise different levels of galactose in conjunction with lower dosages of the drug were tested for abilities to augment the opacity formation.

## RESULTS

*a. Toxicity and cataract production.* The survival time of the rats depended on the dose level of busulfan (table 1). The larger amounts produced death in a relatively short time while smaller doses were less lethal or had no effect upon the animals. Doses between 31 and 1,000 mg per kilogram of diet caused death before cataract formation. Those between 7.5 and 20 mg per kilogram of diet produced cataracts and growth impairment while those of 5 mg and lower had no significant effects even after 26 weeks on test.

At the end of 84 days the rats receiving 10 mg of busulfan per kilogram of diet were autopsied. In general the animals appeared slightly paler in skin color and somewhat smaller than their untreated counterparts. When the cataracts were observed under slight magnification (10 $\times$ ) it was seen that both the lens and lens epithelium were opaque with a diffuse milky appearance. There was an increased vascularity of the iris which became quite apparent in front of this opaque background. It was also observed that the femurs were more brittle than those of the controls and the epiphyses were not entirely closed or calcified.

Individual hematological values including blood sugar determinations were obtained from the animals receiving the dose of 10 mg of the drug per kilogram of diet (table 2). It was found that the average blood sugar value for the treated

TABLE 1  
*Cataract formation in relation to growth and food and drug intake of rats*

DOSE OF BUSULFAN	AVERAGE INITIAL WEIGHT	AVERAGE FINAL WEIGHT	AVERAGE SURVIVAL TIME	CATARACT FORMATION	TOTAL FOOD INTAKE	DRUG INTAKE
<i>mg/kg diet</i>	<i>gm</i>	<i>gm</i>	<i>days (range)</i>		<i>gm/rat</i>	<i>mg/kg body wt./day<sup>1</sup></i>
1,000.	47.7	37.0	7.8 (7-9)	none	4.9	16.9
500.	47.8	37.3	8.9 (7-12)	none	14.5	21.8
250.	48.3	42.0	12.5 (12-13)	none	37.6	17.9
125.	48.0	49.0 <sup>2</sup>	15.0 (14-16)	none	77.1	13.1
62.5	51.0	80.0	17.1 (11-20)	none	85.1	3.9
31.3	50.9	100.0	25.3 (20-33)	none	179.	2.2
20.0	45.1	188.8	50.4 (28-70)	3 after 8 wks. <sup>3</sup>	949.	1.6
15.6	50.4	206.6	Killed at 84 days (51->84)	4 after 9 wks. <sup>4</sup>	990.	0.890
10.0	45.3	252.8	Killed at 84 days	1 after 10 wks. <sup>5</sup>	1,155.	0.544
Controls	48.0	313.2	Killed at 84 days	none	1,305.	none
7.5	48.9	292.2 <sup>6</sup>	Killed at 182 days	2 after 11 wks. <sup>6</sup>	2,506.	.353
5.0	48.6	320.8	Killed at 182 days	none	2,814.	.241
2.5	48.1	362.2	Killed at 182 days	none	2,924.	.111
Controls	48.3	353.6	Killed at 182 days	none	2,821.	none

<sup>1</sup> Based on the final body weight.

<sup>2</sup> These rats gained up to an average of 55 gm in a week before losing weight and then dying. Animals on lower dosage likewise gained slightly before losing weight and dying.

<sup>3</sup> Cataracts developed in the eyes of three remaining rats after 8 weeks. All rats died before 84 days.

<sup>4</sup> Cataracts developed in the eyes of 4 of 5 rats remaining after 9 weeks. Five other rats died before 84 days.

<sup>5</sup> Cataracts developed in the eyes of 4 of 9 rats remaining after 10 weeks. One rat died before 84 days.

<sup>6</sup> One death at 20 weeks. The final weight value is significantly different from that of the controls, the "t" value being 3.64.

animals was 122.2 mg per 100 ml of blood as compared to that of 106.2 mg for the controls. This was a significant difference, since the "t" value corresponded to  $P = 0.05$ . Hemoglobin, red and white cell counts were lower in the test animals but cataract formation did not appear to be related directly to these lower values. The differential count revealed a lymphocytosis, and bone marrow studies likewise revealed a picture of anemia with definite lack of completion of the hemopoietic

TABLE 2

*Blood studies of rats receiving 10 mg of busulfan/kg diet for 84 days*

FINAL BODY WT.	BLOOD SUGAR	HEMOGLOBIN	RED BLOOD CELLS	WHITE BLOOD CELLS
gm	mg/100 ml	gm/100 ml	$\times 10^6/\text{mm}^3$	$\times 10^3/\text{mm}^3$
269 <sup>1</sup>	135	9.6	2.67	9.85
246	120	11.4	4.62	6.90
239 <sup>1</sup>	124	12.9	5.28	7.05
227	127	11.8	6.74	9.80
228	111	15.1	6.75	14.50
227	120	3.5	1.09	2.90
287	113	14.0	6.07	7.35
288	120	12.8	4.81	10.00
259 <sup>1</sup>	120	3.0	—	—
293 <sup>1</sup>	132	13.8	6.16	12.10

Controls: Average values with ranges for 9 animals.

297 (262-331)	106 (83-131)	15.0 (14.4-16.4)	7.86	15.55
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<sup>1</sup> Cataracts.

cycle. The presence of basophils in large numbers in 6 of the marrow examinations may be considered a response very similar to that found in x-ray therapy.

Histological sections of the spleen and lymph nodes showed necrosis in focal areas and numerous regions of extramedullary hematopoiesis. The glomeruli of the kidney appeared normal but necrosis was present in the epithelium of the proximal convoluted tubules and in the loops of the tubules. The liver was surprisingly normal.

The eye sections showed the development of cataracts from the very first epithelial proliferation in the lens to mature opaque types. The earliest pathological changes appeared to



be an increase in the mitoses of the lens epithelium especially at the equatorial portion. This was followed by vacuolization in the subcapsular cortex, anteriorly, and perinuclear vacuolization. Finally, diffuse feathery opacities were found subcapsularly, anterior and posterior, as well as perinuclear.

*b. Non-reversibility of busulfan cataracts.* In the two groups of rats fed diets containing 15 or 20 mg of busulfan per kilogram of diet the cataracts developed within 8 to 9

TABLE 3  
*Non-reversibility of cataracts*

DOSE OF BUSULFAN	AV. INITIAL WT. <sup>1</sup>	8 WK WT.	TOTAL 8 WK. FOOD INTAKE	DRUG IN- TAKE <sup>2</sup>	AV. SUR- VIVAL TIME	CATA- RACT FORMA- TION	NO. OF CATA- RACTS REVERSED BY CON- TROL DIET
mg/kg diet	gm	gm	gm/rat	mg/kg body wt/day	days		
15	40.1	205.5	712	0.93	> 147 <sup>3</sup>	10 in 63 days	0
20	39.9	163.7	593	1.29	75	8 in 63 days <sup>4</sup>	0
Controls	48.3	237.8	793	—	> 147	none	—

<sup>1</sup> Ten rats per group.

<sup>2</sup> For a period of 8 weeks.

<sup>3</sup> Two animals died after being placed on the drug free diet following cataract formation. Eight were autopsied after 147 days on experiment. No cataract regression could be seen during the last 84 days on the drug free diet.

<sup>4</sup> Two animals died before cataract formation and the remaining 8 died within 30 days after being placed on the drug free diet.

weeks. During the subsequent realimentation period with the animals receiving no drug, those on the 20 mg dose all died within an additional 4 weeks. Those on the 15 mg level survived and resumed a slow growth but no regression was noted in the cataracts during the additional 12 weeks on the control diet (table 3).

*c. Attempts to prevent cataract formation with dietary and other biological supplements.* From the results listed in table 4 it could not be concluded that any of the supplements

tested were active in preventing cataract formation or early death when fed at the level of 20 mg per kilogram of diet. Only the antibiotic, oxytetracycline,<sup>5</sup> gave some indication of slightly prolonging life and producing better growth.

With a lower level of busulfan, 10 mg per kilogram of diet, it appeared that of the supplements added only cod liver oil, a vitamin mixture, thiourea,  $\beta$  pyridylcarbinol tartrate, hydrocortisone and a vegetable oil-casein diet prevented cataract formation. However, deaths occurred in the groups receiving the  $\beta$  pyridylcarbinol and hydrocortisone during the initial 16-week period (table 5). When the dose was increased at that time to 20 mg per kilogram of diet only the two fatty type diets continued to afford protection for 8 additional weeks to all of the original animals. Although the fat increased the caloric content per unit weight of diet and thereby reduced the amount of food eaten the actual drug consumption was still within the range that produced cataracts when the fat content was only that of the Fox Chow diet. The 30% galactose supplement displayed its usual cataractogenic activity in this test and the thyroxin at the 100 mg dosage was especially lethal.

The protective action of fat was verified by using an even more critical level of busulfan, 15 mg per kilogram of diet, from the start of the experiment (fig. 1). Cod liver oil prevented the appearance of cataracts for 21 weeks even though the animals consumed 711  $\mu$ g of busulfan per kilogram of body weight per day. However, two of the 10 animals died before the end of the test. One of the rats receiving the corn oil diet was autopsied after it appeared to have a slight opacity at the end of 8 weeks on test. The remaining 9 exhibited no cataract formation. The busulfan intake for this group was 651  $\mu$ g per kilogram of body weight per day, well within the range for cataract production. At the end of the 21-week period one animal in each group had become quite pale indicating depressed hematopoietic activity. An equivalent amount of vitamins A and D found in the cod liver oil was given as a

<sup>5</sup> "Terramycin" Brand Oxytetracycline, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

TABLE 4  
*Supplements given in attempts to prevent cataract formation or death*  
 (20 mg Busulfan per kg diet, 5 rats per group)

SUPPLEMENT/KG DIET	INITIAL WT.	WT. OF SURVIVORS AT 8 WK.	SURVIVAL TIME	NO. OF RATS WITH CATARACTS <sup>1</sup>	CALCULATED TOTAL 8 WK. FOOD INTAKE	DRUG INTAKE
	gm	gm	days		gm/rat	mg/kg body wt. (8 wk.)/day
Armour liver extract No. 2, 10 gm	45.2	161.6	55.8 (40-84)	1	550.	1.21
Salt mix No. 2, 5 gm	45.6	159.3	51.0 (40-61)	2	557.	1.25
Yeast, autolysate Conc. No. 8 (Nat. Yeast Corp.), 5 gm	46.4	183.0 <sup>2</sup>	33.4 (26-54)	- <sup>2</sup>	567.	1.15
Adenine sulfate, 200 mg	46.2	177.0	44.0 (26-59)	1	502.	1.01
Desiccated whole liver, 50 gm	41.0	163.3	60.8 (26-96)	1	565.	1.23
Oxytetracycline, 500 mg	40.4	211.5	79.4 (42-117)	4	690.	1.16
Riboflavin, 250 mg	40.8	148.0	60.8 (43-87)	1	518.	1.25
Vitamin supplement, <sup>3</sup> 50 ml	39.8	164.3	63.8 (40-98)	2	598.	1.28
DL-Methionine, 500 mg	40.2	163.6	58.8 (44-79)	2	545.	1.18
Cod liver oil, 50 ml	40.6	162.4	57.2 (56-70)	4	652.	1.43
Nicotinic acid, tryptophan and ascorbic acid, 500 mg each	46.0 (3 rats)	151.0	61.0 (54-65)	-	527.	1.25
Vitamin B <sub>12</sub> , 1 mg	49.0	150.0 <sup>2</sup>	30.4 (13-47)	- <sup>2</sup>	395. (43 days)	1.25
Estradiol, 10 mg	48.0	125.0	67.8 (47-83)	4	488.	1.39

<sup>1</sup> All other rats died before cataract formation.

<sup>2</sup> All rats died before 8 weeks.

<sup>3</sup> Vitamin supplement:

2-methylanthraquinone	0.01 gm	Choline chloride	50.00 gm
Calcium pantothenate	1.00 gm	Biotin	0.01 gm
Thiamine chloride	0.25 gm	Inositol	2.00 gm
Riboflavin	0.25 gm	Water	100.00 ml
Pridoxine HCl	0.10 gm	Ethyl alcohol	to 500.00 ml
Nicotinamide	2.00 gm		

TABLE 5

Supplements given in attempts to prevent cataract formation or deaths  
 (50 mg busulfan/kg diet for 16 weeks followed by 4 or more weeks of a 20 mg/kg diet dosage—5 rats per group)

Supplement/kg diet	10 mg busulfan/kg diet for 16 weeks					Subsequent 20 mg busulfan/kg diet for 4 weeks				
	INITIAL WT.	Wt. and No. of survivors at 16 Wks.	Deaths during period	Cataracts formed	Drug intake $\mu\text{g/kg body wt./day}$	Wt. and No. of survivors after 4 additional wks.	Deaths during period	Cataracts formed	Drug intake $\mu\text{g/kg body wt./day}$	
	gm	gm				gm				
Liver powder, <sup>1</sup> 50 gm sodium desoxy-nucleic acid, 1 gm	18.6	287 (4)	0	1	466	290 (2)	0	2	879	
Amino acid mixture, <sup>2</sup>	17.0	280 (4)	0	1	488	265 (2) *	0	2	904	
Oxycetracycline HCl, 500 mg	17.2	297 (4)	0	1	470	283 (4) *	0	0	899	
(+)-Galactose, 30%	17.0	218 (4) (91 days)	5	5	611	...	.	.	...	
E-radiol, 10 mg	46.8	297 (4)	0	1	578	215 (3)	0	1	1,136	
Methyl testosterone 100 mg	47.8	237 (4)	0	1	552	255 (1)	0	3	801	
Cod liver oil, 100 ml	49.0	300 (5)	0	0	414	315 (5)	0	0	870	
Vitamin mixture <sup>3</sup>	47.0	295 (5)	0	0	490	302 (4)	0	4	976	
Thiourea, 50 mg	45.8	307 (5)	0	0	468	288 (5)	2 in 5th wk.	1 in 5th wk.	866	
$\beta$ Pyridylcarbinol tartrate, 50 mg	46.4	304 (3)	2	0	475	273 (2)	1 and 1 in 5th wk.	0	869	
Hydrocortisone, 2 mg/kg body wt./day-s.c.	46.0	288 (1)	1	0	490	285 (4)	0	2 in 6th wk.	853	
Vegetable oil, <sup>4</sup>										
Casim diet	46.0	352 (4)	1 in 2 wks.	0	305	334 (4)	0	0	513	
Thyroxine, 100 mg	46.2	160 (1) (79 days)	4	1	1,185	.	.	.	...	
Cholesterol, 1 mg	46.8	316 (2)	0	3	469	315 (1)	0	1	1,199	
BAL, <sup>5</sup> 5 mg/kg body wt./day	46.6	282 (2)	0	3	485	285 (2)	0	0	1,004	

\* Rats were autopsied as soon as cataracts became evident.

<sup>1</sup> De-siccated whole liver no. 35 Wilson & Company, Chicago, Illinois.

<sup>2</sup> 2 gm choline chloride, 500 mg DL methionine and 100 mg of glutathione, nicotinic acid amide, L-cystine and L-cysteine-HCl per kilogram of diet.

<sup>3</sup> All diet within 1 more week.

<sup>4</sup> 500 mg riboflavin, 500 mg ascorbic acid, 1 mg vitamin B<sub>12</sub> and 50 ml of vitamin supplement (table 6) per kilogram of diet.

<sup>5</sup> 20% vegetable oil—20% casein diet, Nutritional Biochemicals Corp., Cleveland, Ohio.

<sup>6</sup> This death at two weeks could not be considered as due to drug action.

<sup>7</sup> British Anti-Lewisite or diisopropyl, administered by subcutaneous injection.

concentrate to another group but no inhibition of cataract formation was obtained. Only one rat survived for more than 12 weeks without cataract formation in the group receiving drug alone in the Fox Chow diet. These observations are summarized in table 6.

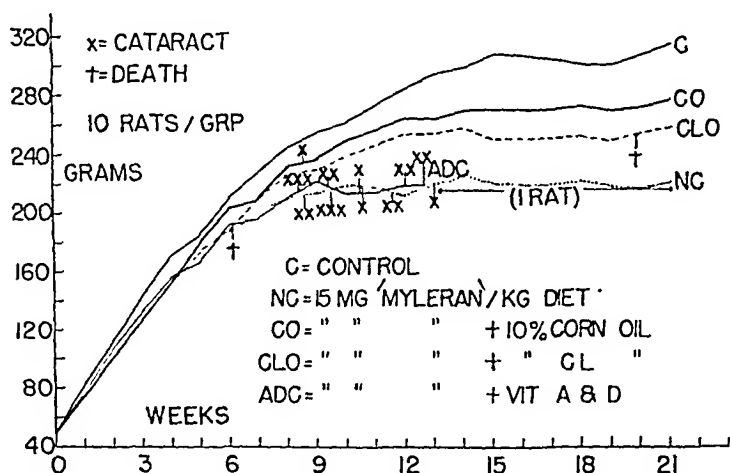


Fig. 1 Effect of 10% corn and cod liver oil on toxicity and cataract formation with busulfan.

TABLE 6

*Prevention of cataracts with fat supplements (15 mg busulfan per kg diet)*

*10 rats/group*

SUPPLEMENT	TIME	DEATHS	CATARACTS	DRUG INTAKE
	days			$\mu\text{g/kg body wt./day}$
Corn oil, 10%	147	0	1	651
Cod liver oil, 10%	147	2	0	711
Vitamin A and D concentrate	84	0	10	921
Busulfan, 15 mg	84	0	9	958
Busulfan, 10 mg	70	0	4	540

When galactose was added to the test diet only the 20% level caused cataracts when the dose of busulfan was 5 mg per kilogram of diet (fig. 2). At the higher busulfan dose of 10 mg per kilogram of diet both the 10% and the 20% amounts

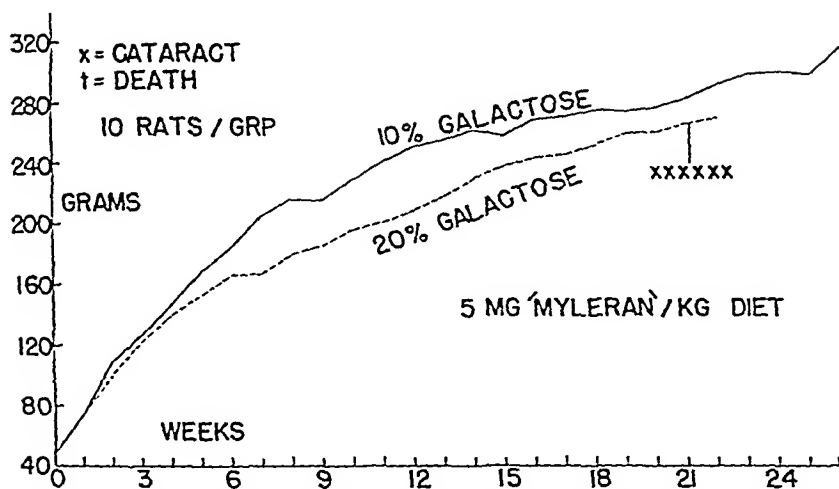


Fig. 2 Augmentation of cataract production with galactose (5 mg busulfan/kg diet).

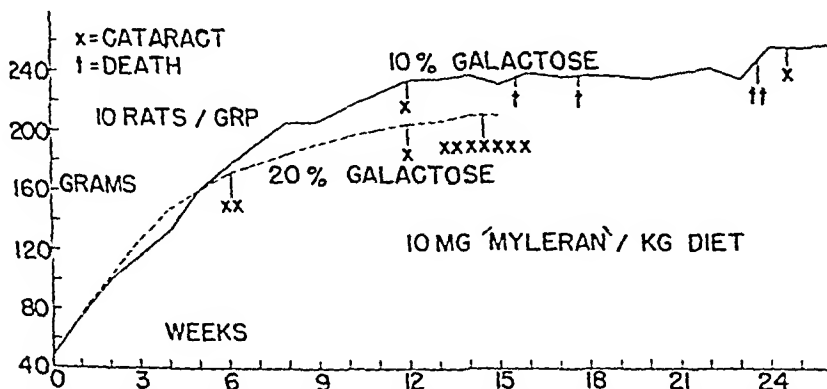


Fig. 3 Augmentation of cataract production with galactose (10 mg busulfan/kg diet).

TABLE 7

*Effects of combinations of galactose and busulfan (10 rats/group)*

BUSULFAN	GALACTOSE	TIME	DEATHS	CATARACTS	DRUG INTAKE
mg/kg diet	%	days			mg/kg body wt. day
5	10	182	0	0	234
5	20	154	0	6	208
10	10	182	4	2	206
10	20	104	0	10	649

of galactose augmented cataract formation (fig. 3), the latter value greatly reducing the time required for their appearance. These results are summarized in table 7.

#### DISCUSSION

From the data presented it may be seen that a new chemical tool has been found for use in the study of cataract formation. Doses of busulfan in amounts many times the therapeutic levels used for treating chronic myelocytic leukemia in humans have produced lens opacities in young and growing rats. The actual drug intakes causing cataracts vary between 350 and 1,600  $\mu\text{g}$  per kilogram of body weight per day as compared with the maximum recommended human doses of only some 60  $\mu\text{g}$  per kilogram. Up to the present time no cataracts in humans attributable to the use of this drug have been found. In fact there may be species difference (Bettman, '46; Barnes and Denz, '54) and strain variations (Mitchell et al., '37) with respect to cataract formation.

Although the blood sugar is slightly elevated in the treated animals it does not appear to be great enough to be the cause of cataract formation according to the criteria of Patterson ('53). This form of cataract also does not appear to be the result of any systemic deficiency of amino acids or proteins as discussed by Hall et al. ('48) and Pike ('51) or of vitamins, especially riboflavin, as reviewed by Day et al. ('38). It would seem more logical to class it with substances such as epinephrine (Suden, '40), naphthalene (Fitzhugh and Buschke, '49) and dinitrophenol (Robbins, '44; Bettman, '46) or anoxia (Bellows and Nelson, '44) all of which appear to have a direct action on lens metabolism. The above deficiencies, of course, may also act in this manner by interfering with certain metabolic systems directly in the lens. These possible actions on oxidation processes have been comprehensively discussed by Bourne ('37), Buschke ('43) and Schlaifer ('54). It would be of interest to evaluate the insulin activity in these drug affected animals in order to determine any decrease in the amount of the hormone which might lead to cataract forma-

tion (Patterson, '54, '55a) although the galactose type seems to be independent of insulin level (Mitchell et al., '37). The idea of an allergic type of cataract produced by busulfan should not be overlooked (Bentolila et al., '52). Calcium and thyroxin levels could also be profitably studied (Brand, '50; Giroud and de Rothchild, '51). Lens metabolism studies (Harris et al., '54 and Christiansen and Leinfelder, '52) may likewise be useful in examining opacities produced by busulfan.

The actual cellular development of busulfan cataracts is quite comparable to that caused by diabetes (Patterson, '52), galactose and various toxic agents (Robbins, '44; Buschke, '43) in the peripheral appearance of vacuoles and opacities, whereas the amino acid-deficient types of cataracts usually exhibit initial opacities toward the center of the lens (Day et al., '38).

The prevention of cataracts in itself has been a prolific field for research. The effect of food and drugs has been discussed by Moore ('40). Von Sallmann ('52) has obtained some protection from x-ray damage to rabbit lenses by pretreatment of the animal with cystine, glutathione or thiourea. However, in the present experiment with 1,4-dimethanesulfonxybutane, which has been continuously administered in the diet to the animals, these adjuncts as well as many others failed to change the course of toxicity or cataract production, even though this chemical has been classed as a radiomimetic drug when administered in single doses (Bergel, '55). Fat in the diet has been found to alleviate cataracts in rats (Charalampons and Hegsted, '50; Rodriguez and Krehl, '51; Nieman, '55) and this protection is evident from the above data in busulfan treated rats at the critical dosage levels used. The toxicity of this drug, therefore, must affect the animal in a different manner than that of sodium fluoride in which case the dietary fat actually increases the toxicity (Miller and Phillips, '55).

According to Patterson ('55b) galactose accelerates cataract formation and this action is confirmed in the present experiment. It would likewise be interesting to see if other



members of the cataractogenic sugar family acted in a similar manner with busulfan.

#### SUMMARY

Oral administration of 1,4-dimethanesulfonxybutane in a dose range between 350 and 1,600  $\mu\text{g}$  per kilogram of body weight per day to growing male albino rats depressed hematopoiesis with accompanying cataract formations.

The cataracts were irreversible and resembled those produced by irradiation and certain toxic chemicals such as naphthalene. Although blood sugar levels were slightly elevated it could not be concluded that this was the causative factor for the cataract production.

Among the dietary and other biological substances tested in rats only the fats delayed toxicity symptoms and possibly prevented the appearance of cataracts at critical intake levels of busulfan up to 711  $\mu\text{g}$  per kilogram of body weight per day for 21 weeks. Galactose, on the other hand, augmented cataract production even when the busulfan was given at doses as low as 268  $\mu\text{g}$  per kilogram of body weight per day.

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entirely, on the kinds and amounts of tissues being synthesized and maintained at the time. The work of Osborne and Mendel ('16, '19) presents good evidence for the existence of a large growth requirement and a slight maintenance requirement for lysine. Mitchell ('47) found that diets in which lysine-deficient cereal proteins were the nitrogen source had higher biological values for the mature rat than for the growing rat. Mitchell ('50) showed that beef muscle, casein and peanut flour (all deficient in methionine-cystine) had lower biological values for the mature rat than for the growing rat. He explained the difference as due to the sustained requirement of the constituents for keratin synthesis (the sulfur-containing amino acids, principally cystine) coupled with the decreased total amino acid requirements in the mature rat as compared to the growing rat. Womack et al. ('53) found that cystine could meet approximately three-fourths of the total requirements for the sulfur-containing amino acids in the mature rat, whereas in the young rat Womack and Rose ('41) reported that only one-sixth of the requirement for the sulfur-containing amino acids could be met by cystine. This indicates a decreased requirement for methionine as age progresses coupled with an increased requirement for cystine, evidenced by the added ability to utilize preformed cystine in the diet at the advanced ages.

In order to establish an amino acid requirement for growth of a given species of animal at a given period of its life-span it seems advisable to determine simultaneously the minimum amount of the amino acid in a diet which carries the minimum amount of protein necessary just to promote maximum nitrogen retention (Mitchell, '43). In the case of the adult animal it seems advisable to measure simultaneously the minimum amount of the amino acid contained in a diet with a protein content just necessary to promote nitrogen equilibrium.

The investigations to be reported here were initiated to develop mathematical means of assessing protein and amino acid requirements precisely at different ages in order to

determine whether the requirements of the sulfur-containing amino acids change significantly in relation to the other amino acids with changes in the age of the animal.

#### GENERAL EXPERIMENTAL PROCEDURES

Three experiments were conducted. The first was a rat growth experiment in which requirements for dietary protein and sulfur-containing amino acids were estimated from daily rates of carcass deposition of the nutrients of interest and estimates of maintenance requirements for animals aged from weaning to 102 days. The second (young rats) and third (mature rats) experiments employed the nitrogen balance technic and provided by statistical means estimates of requirements of the nutrients of interest while their dietary concentrations were being simultaneously varied.

All experiments were conducted in an air-conditioned room maintained at  $78 \pm 2^\circ$  F. The animals were housed in individual screen bottom cages except when they were undergoing collection periods, when appropriate metabolism cages were used.

The diets employed were of the semi-synthetic type. The protein source was Labeo casein. The nitrogen analysis was done by the Kjeldahl-Wilfarth-Gunning method (A.O.A.C., '50); the ether extract, by a 48-hour extraction of the dry sample with petroleum ether (Skellysolv F) in a Soxhlet apparatus; the moisture determination, by heating for 5 hours at  $105^\circ$  C.; the methionine assay, by the method of Lyman et al. ('46); and the cystine determination, by a modification of the Lyman methionine procedure where 400 mg of DL-methionine were substituted for 200 mg of L-cystine per liter of double-strength medium.

*Experiment 1.* This experiment was planned to estimate body deposition of nitrogen and the sulfur-containing amino acids in rats growing normally on an adequate diet. Thirty male weanling albino rats were given the experimental diet

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# THE EFFECT OF AGE ON THE PROTEIN AND METHIONINE REQUIREMENTS OF THE RAT<sup>1,2</sup>

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That the protein requirement for maximum growth decreases with increasing age and body weight has been known for some time. Mitchell et al. ('36-'37) found that in order to induce maximum nitrogen retention in 40- to 50-lb. pigs confined in metabolism cages more than 26% of dietary protein was required; for 100-lb. pigs, about 22%; for 150-lb. pigs, about 17%; and for 175- to 200-lb. pigs, about 15%. Reber et al. ('53) found that a ration containing 41% casein produced maximum weight gains and feed efficiency for the very young pig, but as the pigs approached 8 weeks of age 20% casein was used as efficiently as higher levels. Since the protein requirement is a summation of amino acid requirements, the latter must also decrease with increasing age.

It appears highly probable that proportions existing among amino acids in the protein requirement change as the animal reaches mature size, because the requirements for the individual amino acids will depend to a large extent, if not

<sup>1</sup>The data reported in this paper are taken from a thesis of the same title, prepared under the guidance of Dr. H. H. Mitchell, and submitted by E. W. Hartsook in the year of 1954 to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.

<sup>2</sup>An unabridged version of this paper received one of the Guba Foundation's Awards for 1954-55 for research relevant to the problems of ageing.

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ad libitum. The percentage composition of the experimental diet was: protein<sup>1</sup> 20, corn oil<sup>2</sup> 12, cerelese 40, sucrose 19.9, vitamin premix (Hartsook and Johnson, '53) 0.5, mineral mix 446 (Spector, '48) 4, woodflock 2, and choline 25% dry mix 1.6. The vitamin premix was used throughout all experiments and was found to be adequate as judged by the criteria of normal growth and the absence of any deficiency symptoms.

The food consumption was determined weekly, as was the body weight. When the mean weight of the group of animals had increased from 20 to 25 gm above the mean weight last determined, the two rats that were nearest to the mean weight of the group were etherized, their gastrointestinal tracts were completely emptied, and the carcasses were analyzed for total nitrogen and for methionine and cystine. The assay procedures used resulted in excellent recovery of added methionine (96%) and good recovery of added cystine (80%).

An equation of the type  $W = a - be^{-ct}$ , where  $W$  = mean body weight,  $t$  = age in days,  $e$  is the base of natural logarithms, and  $a$ ,  $b$ , and  $c$  are constants, was fitted to the growth data by the method of least squares.

The equation follows:

$$W = 530 - 828e^{-0.0197t} \quad (1)$$

The above type of equation is the one used by Brody ('45) to describe the self-inhibiting phase of growth. Differentiation of the fitted equation and evaluation of the resulting differential  $dW/dt$  at an age of 40 days indicates a daily rate of gain of 7.4 gm.

<sup>1</sup> Supplied by Labco casein containing 88.46% crude protein ( $N \times 6.25$ ) by analysis, supplemented with DL-methionine of 97.8% purity at the rate of 3.2% of pure methionine. The DL-methionine used throughout this work was supplied by Merck and Company, Inc., Rahway, New Jersey, through the courtesy of Dr. Harold H. Draper. The casein contained 3.2% methionine and 0.25% cystine per 16 gm of nitrogen.

<sup>2</sup> Vitamin A and D concentrate oil (Distillation Products Industries, Rochester New York) and  $\alpha$ -tocopheryl acetate were added to the corn oil so that the final diet contained 2,000 I.U. of vitamin A, 200 I.U. of vitamin D, and 10 mg of vitamin E per 100 gm.

The data for mean daily food consumption at increasing body weights is well described by the quadratic equation

$$F = 15.5 + 0.120 (W - 186) - 0.000201 (W^2 - 40,987) \quad (2)$$

in which  $F$  is the daily intake of food in grams when the rats attained a body weight of  $W$  in grams.

Equations of the type  $Y$  (carcass component in mg) =  $a - be^{-ct}$  (age in days) were fitted by the method of least squares to the data for the nitrogen, methionine and cystine contents of the rats, with the following results:

$$\text{Nitrogen, } Y = 13623 - 27426e^{-0.0001t} \quad (3)$$

$$\text{Methionine, } Y = 1741 - 3583e^{-0.0001t} \quad (4)$$

$$\text{Cystine, } Y = 1535 - 3908e^{-0.0001t} \quad (5)$$

Figure 1 shows the relationship existing between the net N requirement and age for the experimental animals. Entirely similar graphic representations for net methionine and net cystine requirements are omitted for the sake of economy of space. The values upon which the lower (solid) curve of figure 1 is based were obtained by evaluation of equation (3) at values of  $t$  within the range covered by this experiment and at extrapolated values beyond the range studied.

The center curve represents the summation of the lower curve and estimated values for the maintenance requirement. The net maintenance requirements were arrived at as follows: The N requirement was calculated from the data of the metabolism experiment with mature rats to be described below by multiplying the N maintenance requirement of 1.05 mg N/weight<sup>0.75</sup>/day found in that experiment by the calculated weight<sup>0.75</sup> of the rats at the various ages. The requirements for methionine and cystine were calculated by apportioning the maintenance requirement of methionine of 40.05 mg/weight<sup>0.75</sup> (kg)/day, found by Nasset and Anderson ('51), between methionine and cystine in proportion to their concentrations in rat muscle tissue on the fresh weight basis and the weight<sup>0.75</sup> at the particular age. The concentrations of methionine and cystine of rat muscle tissue used for the calculations were 0.475 and 0.221%, respectively, reported by Dunn et al. ('49) and Lee and Lewis ('34).



The upper curve (short dashes) of figure 1 represents the summation of the center curve and estimated values for N lost from the body as shed hair. The amounts of N, methionine, and cystine lost from the carcass as shed hair were estimated by multiplying the body surface area in square centimeters of the rats by a factor of  $\frac{(70 \times \text{gm of component per } 100 \text{ gm of hair})}{307}$ . The

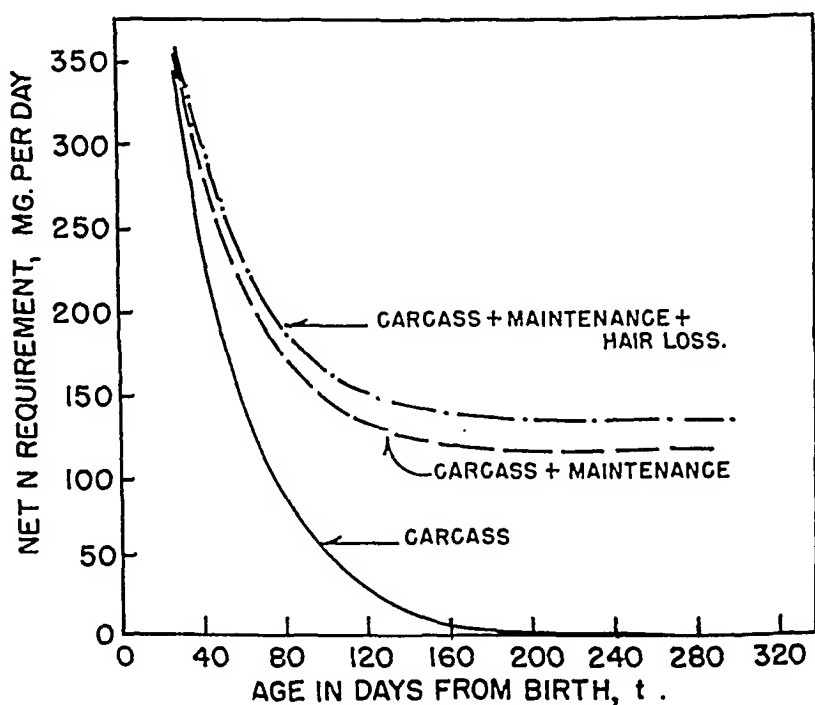


Fig. 1 Relation of net N requirement to age in days from birth of rats fed an adequate diet ad libitum.

figures of 70 and 307 represent the average amount of hair per day recovered by Mitchell ('34) in the feces of rats during periods of ad libitum feeding and the mean surface area in square centimeters of the rats involved, respectively. The figure of 70 mg of hair loss per day, which is the only quantitative figure on hair shedding by rats found in the literature, includes only hair contained in the feces; it is probably

a minimum value. Butcher ('34) found that hair cycles in the albino rat occur approximately every 35 days, with the resting period and the growing stage each being about 17 days in length. According to the data of Smuts et al. ('32) the mean weight of the hair coat of rats fed adequate diets and having a mean body weight of 143 gm and a mean surface area of 246 cm<sup>2</sup> was 2.451 gm. If it is assumed that the entire hair coat is shed during a hair cycle, then the rats of Smuts et al. would have shed  $\frac{2.451 \times 1000}{35} = 70.03$  mg

TABLE 1

*Average daily net requirements of nitrogen, methionine, and cystine by rats at selected ages*

AGE IN DAYS	AVERAGE DAILY NET REQUIREMENTS IN MG/DAY				RATIO OF CYSTINE RE- QUIRED TO METHIONINE REQUIRED	RATIO OF METHIONINE + CYSTINE REQUIRED TO N REQUIRED
	Nitro- gen	Methio- nine	Cys- tine	Methionine + cystine		
30	360	49	54	103	1.11	0.286
38.3 <sup>1</sup>	315	41	46	87	1.12	0.276
50	263	34	38	72	1.12	0.274
75	195	25	29	54	1.16	0.277
100	162	21	25	46	1.19	0.284
200	134	18	24	42	1.33	0.313
300	134	18	24	42	1.33	0.313
342 <sup>1</sup>	134	18	24	42	1.33	0.313

<sup>1</sup> These animals are comparable in age to the animals with values obtained in the metabolism studies.

of hair per day. The rats of Mitchell ('34) were larger than those of Smuts (mean body weight of 206 gm and mean surface area of 307 cm<sup>2</sup>) and therefore should have shed a proportionately greater amount of hair according to the ratio existing between the surface areas of the animals concerned. These calculations confirm in a striking fashion the value used here for the daily loss of hair in the albino rat.

Average daily net requirements for N, methionine, cystine, and methionine + cystine at selected ages, together with the ratios of cystine requirement to methionine requirement and methionine + cystine requirement to N requirement are given in table 1. Values in this table relating to the growth study,

comparable as to age of animals with values obtained in the metabolism studies, have been marked with an asterisk (\*).

A dietary protein requirement may be computed from the net N requirement by multiplying the latter by 6.25 and dividing by mean daily food consumption and a biological value of 90<sup>6</sup> for casein adequately supplemented with respect to methionine. Dietary requirements for the sulfur-containing amino acids may be calculated in the same manner from net requirements. The selected biological value of 90 was based on the values of 89, 96, and 83 found by Mitchell ('24) and Brown ('49) using adult rats and by Kik ('38) using young rats, respectively. That biological values applicable to proteins of a diet are also applicable to their constituent amino acids is subject to some doubt, but since definite information in regard to the question is lacking, such calculation was made and submitted for what it may be worth. The resulting dietary requirements of protein and of methionine + cystine appear graphically in figure 2, and there the relationships of such dietary requirements to age are shown to be exponential in character.

*Metabolism experiments.* The experimental diets contained uniformly 5% lard, 1.5% cod-liver oil, 0.5% wheat germ oil, 10% sucrose, 39% corn starch, 4% salts no. 446, 0.5% NaCl, 0.5% vitamin premix, 1.6% choline 25% dry mix, and 2% woodflock. The diets were completed by the addition of varying amounts of protein, cerelose, and corn oil. The protein was included in the experimental diets at levels varying from 1.6 to 4.5% in the case of the mature rats of experiment 3 and at levels varying from 10 to 22% in the case of the young rats of experiment 2. In both experiments each dietary level of protein was supplemented with DL-methionine at the rate of 0, 2, 4, and 6% of the

<sup>6</sup>The use of the same biological value for casein adequately supplemented with methionine for diets through a range of protein levels from 10 to 22% may be justified on the assumption that at the higher levels the absorbed protein used for maintenance and growth is still utilized to the extent of 90%. That portion not so utilized will be deaminated and the nitrogen excreted in the urine.

protein. The additional casein in the higher protein diets was always added at the expense of cerelese. Sufficient corn oil was added so that the total of corn oil and the ether extractives of casein amounted to 5% of the diet, thereby maintaining the fat content of the diets at 12%. Standardization diets contained laboratory-prepared whole egg protein, petroleum-ether-extracted at low temperature, as the sole source of protein.

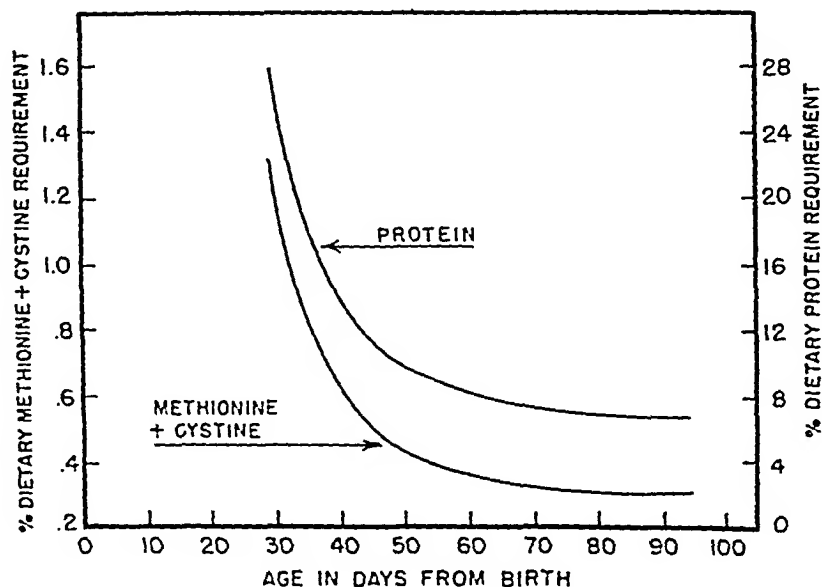


Fig. 2 Relation of the dietary protein and dietary methionine + cystine requirements to age in days from birth of rats (experiment 1) fed an adequate diet *ad libitum*.

Since in these experiments it was necessary that the responses of rats of differing body weights be pooled, a unit was needed which would allow responses of animals or groups of animals of varying body weights to be equitably compared. The unit selected was N balance (mg)/body weight (gm) raised to the 0.75 power ( $N \text{ bal. } \text{wt.}^{0.75}$ ).

Mathematical functions best describing the data at hand were used in interpreting the experimental results. Regres-

sion equations, linear, exponential, or quadratic, were fitted by the method of least squares, either in the original form or after appropriate transformation, using the method of weights when necessary. Fitting regression equations by the method of weights is a modification of a method given by Goulden ('52). The calculation of variances of dependent and independent variables followed the procedures of Snedecor ('46) or followed derivations given by Hartsook ('54).

*Experiment 2. Estimation of requirements for the young rat.* Four lots of 16 weanling rats each were received at weekly intervals. The animals were all 22 days of age on the day received and upon receipt were assigned randomly to each of 4 treatments and were fed an 8% protein (dried defatted whole egg) diet for a 7-day period, a 4% egg protein diet for a 14-day period, and either diets containing 10, 14, 18 or 22% casein for a 14-day period. Diets within each series contained either 0, 2, 4, or 6% of DL-methionine supplement, expressed as a percentage of the protein. During the last 7 days of the experimental periods, excreta were collected.<sup>7</sup> During all periods the animals were given daily weighed amounts of diet determined in accordance with equation (2).

In the case of the 10% protein diets, the mean responses to the three highest methionine supplementation levels were shown by an analysis of variance to be significantly greater than the mean response to the lowest level, but were not significantly different from one another (see figure 3). From these findings it was concluded that 10% protein diets could be improved by 2% methionine supplementation (expressed as a percentage of the protein), but not further improved by additional increments of methionine. Therefore, a straight line was fitted by the method of least squares to the first two arrays of data and a horizontal line was fitted to the last two arrays of data. The equations of both of these lines, as

<sup>7</sup> Plans for collection equipment kindly supplied by Dr. Doris H. Calloway, Nutrition Division, Food and Container Institute, Quartermaster Corps, Chicago, Illinois.

well as the points of data, are presented in figure 3. The fit of the regression equations to their respective arrays is highly significant ( $P < 0.001$ ). The  $Y$  and  $Z$  values of the intercept of the fitted lines are  $29.36 \pm 0.32$  mg and  $0.189 \pm 0.015\%$ , respectively.

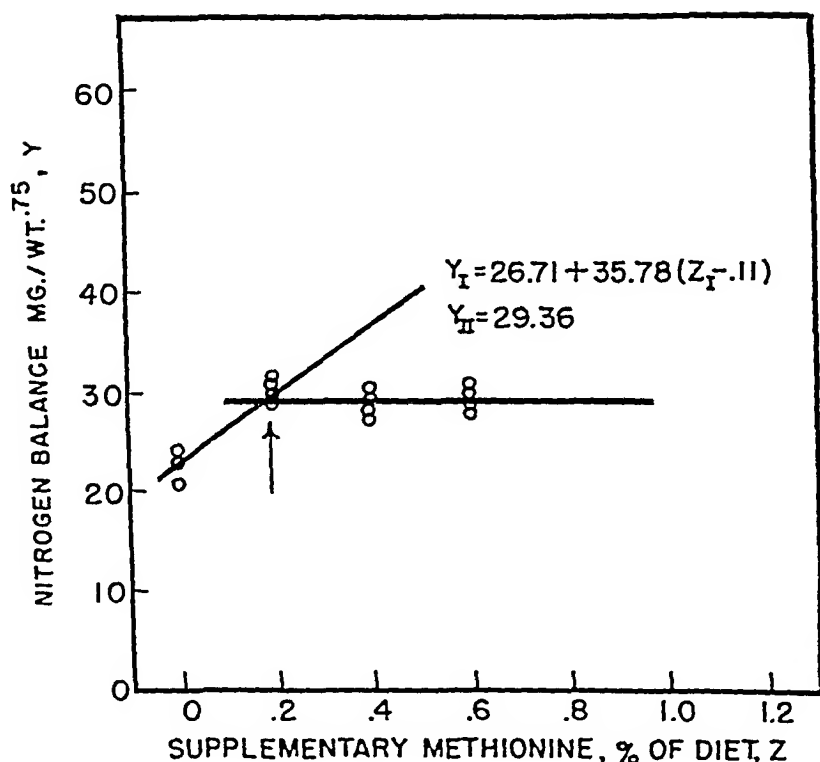


Fig. 3 Relation of N bal./wt.<sup>0.75</sup> to percent supplementary methionine at a 10% level of dietary protein. In figures 3, 4, 5, 6 and 7 the significance of the point(s) on the curve(s) indicated by the arrow(s) and its (their) coordinates is discussed in the text.

In the case of the 14% protein diets, it was found that the third array of data was not consistent with the other arrays (see figure 4). Moreover, the second and 4th arrays were found to be on the general plateau of N balances found for the entire experiment; this made it appear highly probable that the third array should also be on the plateau. The

first array was significantly lower ( $P < 0.01$ ) than the second, while the second array was not significantly lower ( $0.1 < P < 0.2$ ) than the 4th. An exponential function of the type  $Y = a - be^{-cz}$  fitted to all 4 arrays of data did not yield a satisfactory fit (i.e., the regression did not account for a significant portion of the sum of squares of deviations of  $Y$  from its mean), but a similar function fitted to the first, second, and 4th arrays of data did yield a satisfactory fit. The third array was, therefore, eliminated from further con-

TABLE 2

*Maximum values of N balance (mg)/weight<sup>0.75</sup> (Y), minimum methionine supplementation (Z) resulting in maximum N balances, and percentages of dietary protein (X) found with young rats fed methionine-supplemented casein diets*

% PROTEIN (X)	MAXIMUM N BALANCE (mg)/ WEIGHT <sup>0.75</sup> (Y)			MINIMUM METHIONINE SUPPLEMENTA- TION (EXPRESSED AS A PERCENTAGE OF THE DIET) RESULTING IN MAXIMUM N BALANCES (Z)		
	N Balance	Variance	Weight	% Methio- nine	Variance	Weight
10	29.36 <sup>1</sup>	0.10	9.99	0.189 <sup>1</sup>	0.000229	4,367.61
14	38.74 <sup>1</sup>	0.91	1.10	0.488 <sup>2</sup>	0.116	8.64
18	39.04 <sup>1</sup>	0.52	1.94	0.537 <sup>2</sup>	0.0367	27.23
22	38.68 <sup>1</sup>	0.38	2.65	0	—	—

<sup>1</sup>  $P = 0.001$ .

<sup>2</sup>  $0.1 < P < 0.2$ .

<sup>3</sup>  $P = 0.05$ .

sideration, and the latter fitted equation is given in figure 4. Brody ('45) considers the practical maximum of such a function to be reached at 98% of the value of the asymptote, and since at approximately this value of  $a$  the value of  $Z$  loses significance, 98% of the asymptotic value was taken to be the maximum N bal./wt.<sup>0.75</sup> and the corresponding value for supplementary methionine was calculated. These values of  $Y$  and  $Z$  with their standard deviations are, respectively,  $38.7 \pm 0.97$  mg and  $0.49 \pm 0.34\%$  of the diet. The value of  $Y$  was highly significant (table 2), but  $Z$  was found only to border on significance. Since weighted values were to be

used in the following steps of the interpretation, the value of  $Z$  was accepted as the best one available.

The method of interpretation of data for the 18% protein diets was entirely similar to that for the 10% protein diets and for the sake of brevity is not presented graphically. The

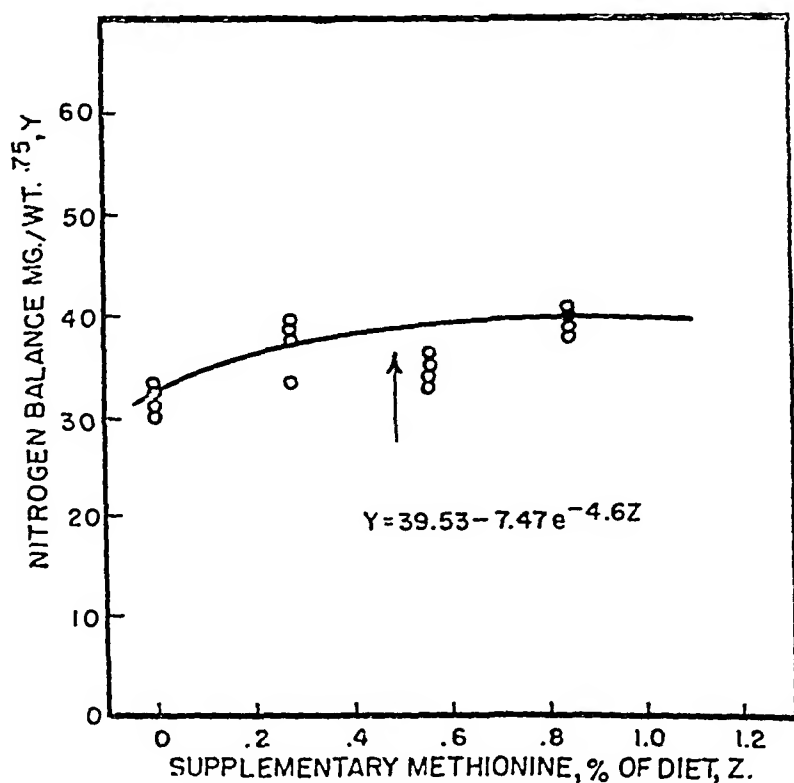


Fig. 4. Relation of  $N$  bal./wt.<sup>.75</sup> to percent supplementary methionine at a 14% level of dietary protein.

intercept values for  $Y$  and  $Z$  appear in table 2, where it will be noted that the value of  $Y$  is significant at the 0.001 level and the value of  $Z$  is significant at the 0.05 level.

The mean responses ( $N$  bal./wt.<sup>.75</sup>) for the 22% protein diets at the various methionine supplementation levels were not significantly different from one another, and the slope of a line passing through the 4 arrays of data was found to be



not significantly different from zero ( $P > 0.8$ ), showing that methionine supplementation of a diet containing this amount of protein is without significant effect on nitrogen balance. The equation of the regression line became  $Y = 38.68$ . The value of  $Y$  was highly significant. The responses,  $Y$ , for the 22% protein diets are not shown graphically.

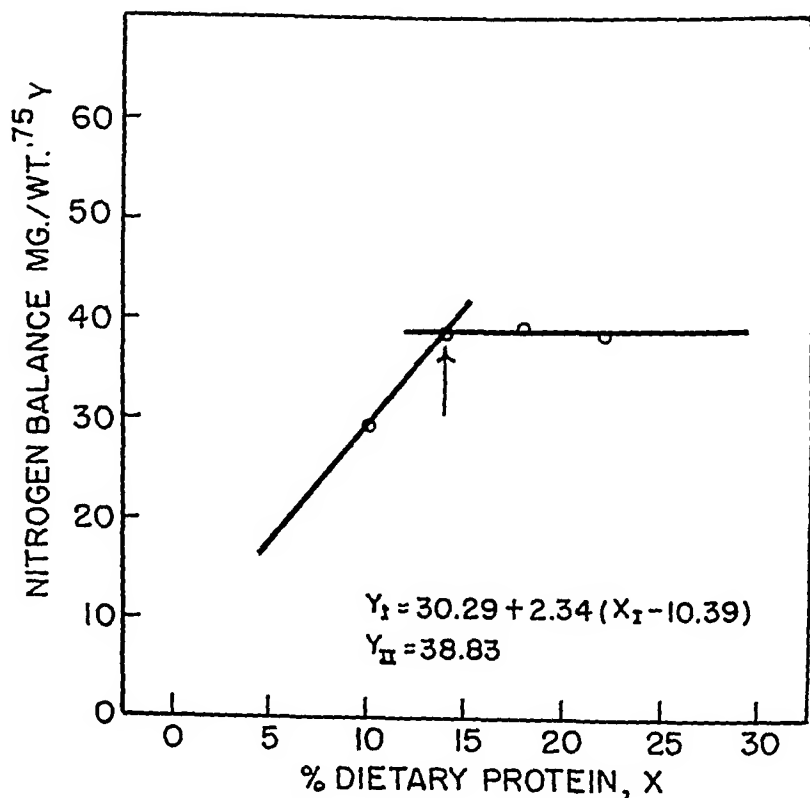


Fig. 5 Relation of maximum N bal./wt.<sup>0.75</sup> determined at various methionine supplementation levels ( $Y_1$ ,  $Y_2$ ,  $Y_3$  and  $Y_4$ ) to percent of dietary protein.

From table 2 it can be seen that the N bal./wt.<sup>0.75</sup> was not improved significantly when the dietary level of protein was increased above approximately 14%. Short of having additional information from many rats, the best approximation of the protein requirement is apparently the point of

intersection of lines drawn through the first two points and the second two points, respectively. The variance of each maximum N bal./wt.<sup>0.75</sup>,  $Y$ , for the respective protein levels was converted to a weight by taking the reciprocal of the variance; these weights were used in fitting a regression line to the first two points, and in obtaining a mean value for the last two points. Plots of these two lines, together with the optimum values of  $Y$  previously calculated, appear in figure 5. The point of intersection has the coordinates:  $X_R$  (percentage of dietary protein) = 14.04 and  $Y_R$  (N bal./wt.<sup>0.75</sup> at the value of the protein requirement) = 38.83. The variance and standard deviation of  $X_R$  were found to equal 0.21 and 0.46, respectively.

In table 2 are also found the variances of the optimum levels of methionine supplementation at each dietary protein level. These variances were converted to weights in the same manner as previously described for protein. An exponential function,  $Z = 0.54 - 52.38e^{-0.5x}$ , where  $Z$  = percent of methionine supplementation expressed as a percentage of the diet and  $X$  = percent of dietary protein, was fitted to the first three points of data by the method of weights (see figure 6). The  $Z$  values upon which the fitting of the equation was done were the minimum levels determined in the interpretations of the first three series of experimental diets, and the  $X$  values were the protein percentages of the individual series of diets. The minimum level of methionine supplementation resulting in maximum N balances was taken as the  $Z$  intercept of the protein requirement value, 14.04, and is designated as  $Z_R$ , the requirement for supplementary methionine.  $Z_R$  was found to equal 0.49, and its variance and standard deviation to equal 0.023 and 0.15, respectively.

*Experiment 3. Estimation of requirements for the mature rat.* Twenty-eight adult male rats, weighing from 308 to 401 gm (mean = 361 gm) and approximately 300 days of age, were removed from the stock diet, randomly assigned to 4 groups of 7 animals each, placed on a 16% casein diet for a 7-day period, given the experimental diets for a 14-day

period, given a 3.9% protein (dried defatted whole egg) diet for a 14-day period, and finally given the experimental diets for an additional 14-day period. The experimental diets contained three levels of casein, 0 (low egg-protein diet), 4.0 to 4.5% and an intermediate level ranging from 1.6 to 2.0%. At each protein level, except the 0 level, 0, 2, 4, and 6% of DL-methionine supplement (expressed as a percentage of the

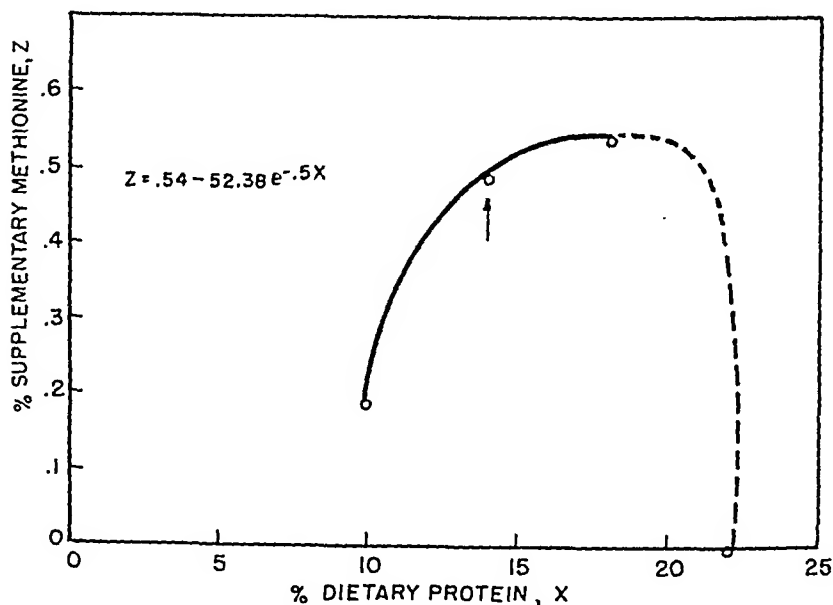


Fig. 6 Relation of minimum methionine supplementation levels ( $Z_1$ ,  $Z_2$ ,  $Z_3$  and  $Z_4$ ) to percent of dietary protein.

casein in the diet) were tested in N balance studies extending over 14 days during the last 7 days of which the excreta were collected.

All rats were given 14 gm of diet per day in all periods, an amount that maintained body weight almost constant. Rats showing symptoms of respiratory disease or refusing in excess of 0.6 gm of food per 7 days were not used in the interpretation of the results of the experiment.

Direct results obtained just prior to the feeding of egg-protein diets, and reversals obtained after such feeding, were analyzed separately.

In interpreting this experiment a modification of a procedure proposed by Melnick and Cowgill ('37) for evaluating the minimum amount of dietary protein necessary for N equilibrium in the adult dog receiving an adequate caloric intake was used. In the work reported here a minimum percentage of dietary protein as well as a minimum amount of methionine supplementation necessary for N equilibrium was desired; therefore, N bal./wt.<sup>0.75</sup> was plotted against percentage of protein in the diet for each level of methionine supplementation for both the direct and reversal periods. The regressions were linear and for each set of observations it was possible to estimate (1) the level of dietary protein necessary for nitrogen equilibrium and (2) the level of methionine supplementation, expressed as a percentage of the dietary protein, required in each case to attain N equilibrium at the lowest protein intake. Values for protein decrease to a minimum and then increase only slightly as the level of methionine supplementation increases from 0 to 6% of the protein.

The course of these curves for direct and reversal periods, shown in figure 7, is well described by the quadratic equations given in the figure. The decrease in  $X$  with changes in  $Z$  from 2 to 4% were highly significant, but the decreases in  $X$  as  $Z$  changed from 0 to 2% were not significant. Hence, the first two values of  $X$  for  $Z=0$  and  $Z=2$  were pooled in fitting the quadratic regression lines to the data by the method of weights. The fitted regression lines are shown in figure 7. In order to determine the point at which  $X$  reached its minimum value for each curve, the first derivative ( $dX/dZ$ ) of the equation was equated to zero and solved for  $Z$ . These values of  $Z$  were substituted in the original regression equations, and corresponding values of  $X$  were calculated. The coordinates of the minima are: for the protein requirement,  $X_{min} = 3.18 \pm 0.10\%$  of the diet, for the methionine require-

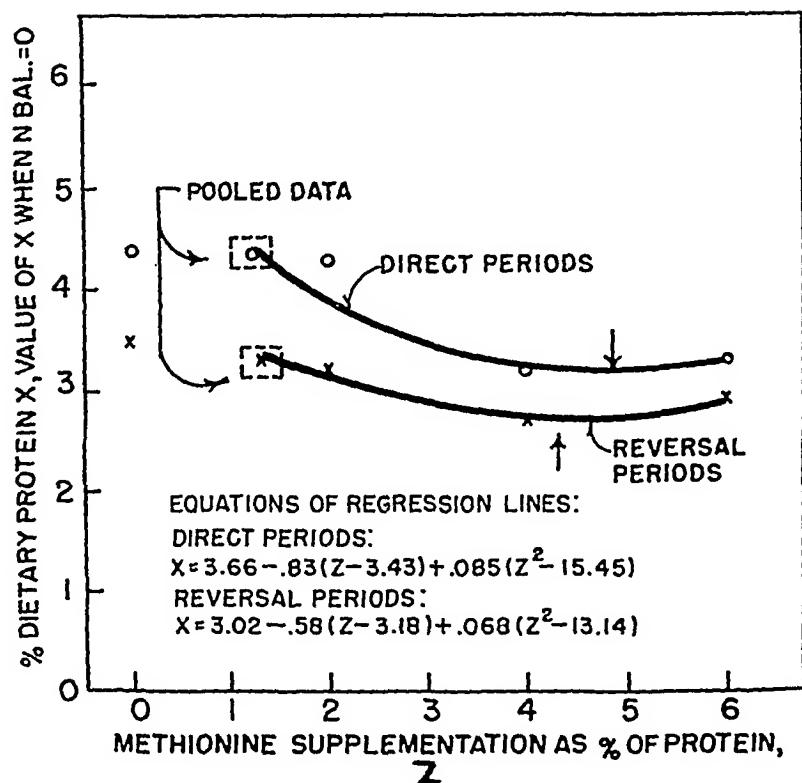


Fig 7 The protein and supplementary methionine requirements of the mature rat. Regression of levels of dietary protein necessary for N equilibrium at the levels of methionine supplementation used on the percent of methionine for mature rats in direct and reversal periods.

TABLE 3

The estimated dietary methionine and dietary methionine + cystine requirements of non-depleted and partially nitrogen-depleted adult rats

COMPONENT	NON-DEPLETED DIRECT PERIODS <sup>1</sup>		PARTIALLY DEPLETED REVERSAL PERIODS <sup>2</sup>	
	% of Protein	% of Diet	% of Protein	% of Diet
Methionine (total)	7.80	0.248	7.80	0.212
Cystine (from casein and total)	0.25	0.008	0.25	0.007
Methionine + cystine (total)	8.05	0.256	8.05	0.219

<sup>1</sup> Indicated protein requirement = 3.18%

<sup>2</sup> Indicated protein requirement = 2.72%.

ment,  $Z_{\min} = 4.85 \pm 0.52\%$  of the protein in the case of the direct periods; and in the case of the reversal periods, for the protein requirement,  $X_{\min} = 2.72 \pm 0.07\%$  of the diet, for the methionine requirement,  $Z_{\min} = 4.31 \pm 0.34\%$  of the protein. The values appended to the above estimates are standard deviations.

The estimates of the methionine requirements in the direct and reversal periods are indistinguishable statistically, but those for the protein requirements are significantly different. This considerable difference in the amount of protein required to achieve N balance during the direct and reversal periods is in agreement with the work of Allison et al. ('49) and of Allison ('51).

In table 3 the requirements found for the sulfur-containing amino acids are expressed both as a percentage of the protein and of the diet for the direct and reversal periods. Since the minimum percentages of methionine supplementation evaluated in the direct and reversal periods were found not to differ significantly, they have been pooled to obtain the entries of columns 2 and 4 of the table.

#### DISCUSSION

In table 4 are assembled data obtained from the metabolism experiments. Tests of significance were run, comparing the experimental values obtained. The difference between protein requirements found in the two experiments was highly significant ( $t = 19.2$ ,  $P < 0.001$ ). The methionine + cystine requirement of young rats was found to be significantly greater than that of mature rats when both requirements were expressed as a percentage of the diet ( $t = 4.8$ ,  $P < 0.001$ ). The total methionine + cystine requirement of mature rats was found to average greater than that of young rats when both requirements were expressed as a percentage of the protein, but the difference between averages was not statistically significant ( $0.3 < P < 0.4$ ).

The dietary protein and methionine + cystine requirements found in the rat growth experiment (experiment 1) are presented graphically in figure 2.

The requirements in terms of dietary concentrations were converted to requirements in terms of milligrams per day by multiplying the former by the daily food consumption. The ratios between methionine + cystine requirement and N requirement (both expressed as milligrams required per day) for the mature and young rats were found to be 0.503 and 0.437, respectively. The ratio of the value for the greater age to the value for the lesser age was  $\frac{0.503}{0.437} = 1.15$ . It will be recalled that the ratios of net methionine + cystine re-

TABLE 4

*Dietary protein and methionine + cystine requirements determined in metabolism experiments with young and mature rats*

AGE OF RATS USED	EXPERIMENT NUMBER	MEAN AGE IN DAYS	MEAN BODY WEIGHT	DAYS (CALCULATED FROM MEAN BODY WEIGHT) <sup>1</sup>	PROTEIN REQUIREMENT (% OF THE DIET)	METHIONINE + CYSTINE REQUIREMENT	
						% of diet	% of protein
Young	2	53	141	38	14.04	0.98	7.0
Mature	3	342	362	81	3.18	0.26	8.0

<sup>1</sup> Using equation (1).

quirement to net N requirement recorded in table 1 for rats of the growth experiment at ages of 342 and 38.3 days were 0.313 and 0.276, respectively. The ratio of the value for the greater age to the value for the lesser age was  $\frac{0.313}{0.276} = 1.13$ . The fact that the two ratios cited stand in excellent agreement is indicative that keratin synthesis assumes an increasingly important role in protein biosynthesis as maturity is approached and latter attained.

#### CONCLUSIONS

The conclusions based on feeding male albino rats diets containing varying levels of casein, 12% fat and 2% bulk, with varying supplements of methionine follow:

1. The protein requirement (casein plus adequate methionine), expressed as a percentage of the diet, decreases in an exponential fashion as age increases from about 14% to about 3.2%.

2. The methionine plus cystine requirement, expressed as a percentage of the diet, decreases in an exponential fashion as age increases from about 0.98 to 0.26%. As a percentage of the protein requirement it increases from 7.0 to 8.0%.

3. Evidence presented (although no individual portion reaches statistical significance within itself) is unanimous in indicating that keratin synthesis assumes an increasingly important role in protein biosynthesis as maturity is approached and later attained.

#### ACKNOWLEDGMENTS

Valuable guidance in the statistical treatment of the many observations secured in these studies was received from Dr. Mark H. Bert and Dr. H. W. Norton.

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# RESISTANCE OF MOUSE TISSUE SULFHYDRYL TO ALTERATIONS BY CHANGES IN DIETARY INTAKE OF SULFUR AMINO ACIDS<sup>1</sup>

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The likelihood of death occurring in mice or rats subsequent to x-irradiation of the whole body may be appreciably decreased by prior administration of various sulfhydryl compounds [see Patt ('53) for references], in amounts sufficient to bring about temporary marked increases in tissue sulfhydryl concentrations (Cronkite, Chapman and Brecher, '51). The present research was initiated to determine: (a) to what extent significant alterations in mouse tissue sulfhydryl could be secured by dietary procedures, and (b) whether the alterations which were secured would be significantly associated with changes in likelihood of death occurring subsequent to whole body x-irradiation.

It should be noted that Smith, Ackermann and Alderman ('52) have reported that the susceptibility of rats to lethal effects of whole body x-irradiation was relatively little affected by considerable variation in their dietary intake of protein (casein) and the sulfur amino acids, cystine and methionine. On the other hand Jennings ('49) has reported that rats kept for about 10 weeks on a very low protein diet were much more susceptible to lethal effects of whole body

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x-irradiation than corresponding control rats. Neither group of workers estimated tissue sulfhydryl concentrations of control and test animals.

## METHODS

### *Chemical*

Each tissue sample was frozen on dry ice immediately on excision and held wrapped in aluminum foil in a deep freeze to time of analysis, not more than 24 hours later. Protein-free metaphosphoric acid extracts were secured and analyzed for non-protein sulfhydryl (NPSH) by the nitroprusside method of Grunert and Phillips ('51), standardized against glutathione. Values obtained are shown in the tables as glutathione equivalents in milligrams per cent. Each tissue extract was prepared using pooled tissues of a particular kind, e.g., liver, obtained from all of the mice or the rat weanlings in a given control or test group.

### *Diets*

Prior to their experimental use, mice were kept on a diet of rat pellets,<sup>2</sup> protein content not less than 20%, which were fed ad libitum with water.

*Nitrogen-containing substances and sulfur amino acid of diets.* The percentages of these substances present in the more important synthetic diets employed are indicated in table 1. Methionine derived from protein was the L form, whereas the free amino acid employed was the DL form. However, Wretland and Rose ('50) have reported that the D and L forms of methionine have equivalent dietary values.

A protein, derived from soy beans,<sup>3</sup> constituted the chief source of nitrogen in diets DA1 to DX3 inclusive (table 1). This protein is markedly deficient in its content of sulfur amino acids; it contains 0.6% of L-cystine and 1.0% of L-methionine according to the manufacturer's analysis. All

<sup>2</sup> Rockland.

<sup>3</sup> Drackett Assay C-1 protein, The Drackett Company, Cincinnati, Ohio.

other essential amino acids except tryptophan are present in amounts which the experiments of Rose ('38) indicate are satisfactory for the growth of rats. Free L-cystine and DL-methionine were added to diets DA1, DA2 and DA3 in amounts judged sufficient to render them adequate in sulfur

TABLE 1  
*Nitrogen substances in certain of the diets*

DIET <sup>1</sup>	PORTION OF THE DIET ESTIMATED PRESENT AS:			
	Soybean protein <sup>2</sup>	Free amino acids		
		L-Cystine <sup>3</sup>	DL-Methionine <sup>3</sup>	Other
	%	%	%	%
<i>Experiments with mice</i>				
DA1	18.8	0.2 <sup>4</sup>	1.0 <sup>4</sup>	0.0
DA2	13.8	0.2	1.0	0.0
DA3	8.8	0.2	1.0	0.0
DD2	13.8	0.0	0.0	1.2 <sup>5</sup>
DD3	8.8	0.0	0.0	1.2
DD4	6.3	0.0	0.0	1.2
DX1	18.8	0.2 <sup>4</sup>	3.0 <sup>4</sup>	0.0
DX2	18.8	1.2	1.0	0.0
DX3	18.8	1.2	3.0	0.0
AA (Control)	0.0	0.25	1.25	18.5
AD (No sulfur amino acids)	0.0	0.00	0.00	20.0 <sup>5</sup>
<i>Experiments with rats</i>				
R (Control)	0.0	0.0	1.0	13.9
R minus methionine	0.0	0.0	0.0	12.9 <sup>7</sup>
R minus valine	0.0	0.0	1.0	11.7 <sup>7</sup>
R minus isoleucine	0.0	0.0	1.0	11.9 <sup>7</sup>
R minus threonine	0.0	0.0	1.0	12.9 <sup>7</sup>

<sup>1</sup> For a more detailed description of diets, see Methods, pg. 195.

<sup>2</sup> Drackett Assay C-1 Protein, The Drackett Products Company, Cincinnati, Ohio.

<sup>3</sup> To estimate total in the diet add the amount contributed by the soybean protein, which contains 0.6% of L-cystine and 1.0% of L-methionine.

<sup>4</sup> This amount was added to make the diets adequate in sulfur amino acids.

<sup>5</sup> L-Glutamic acid was substituted for free L-cystine plus free DL-methionine, to supply nitrogen equivalent to that in the sulfur amino acids of the other diets.

<sup>6</sup> Added in amounts to make the combined sulfur amino acids in excess of nutritional needs.

<sup>7</sup> An equal weight of sucrose was substituted for the omitted amino acid.

amino acid content, and to diets DX1, DX2 and DX3 in amounts judged to be in excess of nutritional needs. Free glutamic acid, 1.2%, was substituted for the sulfur amino acids in sulfur amino acid-deficient diets DD2 and DD3, in order to make these diets equivalent in nitrogen content to the corresponding sulfur amino acid adequate diets DA2 and DA3.

Pure amino acids constituted the only source of nitrogen in diets AA to R-T inclusive (table 1).

Diets AA and AD, fed to mice, contained: L-arginine, 0.5%; glycine, 0.5%; L-histidine, 1.0%; DL-isoleucine, 2.25%; L-leucine, 2.0%; L-lysine, 2.0%; DL-phenylalanine, 3.0%; L-threonine, 0.25%; DL-tryptophan, 0.5%; and DL-valine, 2.5%. Control diet AA also contained 0.25% L-cystine, 1.25% DL-methionine and 4.0% L-glutamic acid, whereas sulfur amino acid-deficient diet AD contained 5.5% L-glutamic acid, but neither cystine nor methionine.

Diets R to R-T inclusive of table 1 were fed to rat weanlings. Control diet R contained: L-arginine, HC1, 0.6%; DL-histidine HC1, 1.1%; DL-isoleucine, 2.0%; DL-leucine, 3.2%; L-lysine HC1, 0.85%; DL-methionine, 1.0%; DL-phenylalanine, 1.5%; DL-threonine, 1.0%; DL-tryptophan, 0.45%; and DL-valine, 2.2%.

Sucrose was substituted for DL-methionine in sulfur amino acid-deficient diets R-S, for valine in valine-deficient diet R-V, for isoleucine in isoleucine-deficient diet R-I, and for threonine in threonine-deficient diet R-T.

*Non-nitrogenous substances in the diets.* Diets DA1 to AD inclusive of table 1, fed to mice, contained: (a) cod liver oil, 2%, as source of vitamins A and D; corn oil, 1%, as source of vitamin E, and lard oil, 5%; (b) pure vitamins, as follows: choline, 0.2%; riboflavin, 0.001%; thiamine, 0.001%; pyridoxine, 0.001%; pantothenic acid, 0.004%; niacin, 0.008%; folic acid, 0.0003%; biotin, 0.00001%; and menadione, 0.00002%; (c) the salt mixture of Hubbell, Mendel and Wakeman ('37), 4%; and (d) sucrose, in amount sufficient to bring the sum total of all dietary constituents to 100%.

Diets R to R-T inclusive of table 1, which were fed to rat weanlings, contained: (a) corn oil, 5%; (b) 1.05%  $\text{NaHCO}_3$ ; (c) pure vitamins, as follows: choline, 0.2%; thiamine, 0.0005%; riboflavin, 0.001%; pyridoxine, 0.0005%; pantothenic acid, 0.004%; and niacin, 0.005%; (d) USP XIV salt mixture, 4.0%; and (e) sucrose, in amount sufficient to bring the sum total of *all* dietary constituents to 100%.

### *Feeding and care of test animals*

Mice were kept in open-mesh wire cages. The daily food consumption by the 4 or 5 mice in each particular cage was estimated as follows: 5 gm per mouse of the appropriate diet was placed in an opal jar of inside diameter 1.75" and height 1.5". This jar was placed inside another opal jar of 3.5" inside diameter and 3.75" height. The weight of the food left in the two jars was determined 24 hours later. The difference in the two weights was used as the measure of the day's food consumption for the mice in that cage. Our experience, and that of experimenters in the Mellon Institute Toxicology Laboratory, Pittsburgh, Pa., has been that mice do not remove and scatter appreciable amounts of food from this double jar arrangement, even when they are confronted with diets which are not palatable.

The mice used were males, 6 to 10 weeks of age.<sup>4</sup> The animals used in any one experiment were segregated into cage-groups in such a way that all of the different groups had practically identical average body weights at the beginning of the experiment. These average weights ranged from 19 to 24 gm.

The procedures for rat weanlings<sup>5</sup> were similar to those for mice, except that the daily food consumption was not estimated.

<sup>4</sup> Carworth Farms.

<sup>5</sup> Holtzman.



### *X-Irradiation of mice*

Prior to x-irradiation, one or two control mice, previously kept on a diet judged adequate in sulfur amino acid content, and one or two test mice, previously kept on a diet judged to be grossly inadequate in sulfur amino acid content, were placed in each of 16 plastic cages, each cage being 7" long, 1" high and 1.5" wide. These cages were piled on top of each other in such a way that the center of each cage was, as nearly as possible, 36" from the point source of the horizontal x-ray beam generated by a 30° angle tube. The Picker x-ray machine employed,<sup>6</sup> was run at 205 KV, 15 ma, using 1.0 mm Al plus 0.25 mm filtration. Victoreen meters indicated an average x-ray dose of 14 r per minute. The estimated whole body x-ray doses estimated as having been employed in the different experiments are shown in table 5.

### EXPERIMENTAL

*Sulfur amino acid-deficient diets.* The data obtained in experiments involving the use of diets deficient in sulfur amino acids have been summarized in tables 2 and 3. In those experiments in which the soybean protein formed the chief source of dietary nitrogen, the lowest of the liver NPSH values secured were for mice which had been placed for *one day only* on a diet deficient in sulfur amino acids. The data indicate that a metabolic adjustment occurred for mice kept on this kind of diet for periods longer than one day. This adjustment was of such a nature that the liver NPSH gradually returned toward normal values, in spite of the continuing severe sulfur amino acid deficiency.

From the percent decreases in liver NPSH associated with the use of sulfur amino acid-deficient diets (last two columns of table 2), it may be concluded that more drastic decreases in liver NPSH were induced by placing mice on a pure amino acid diet *devoid* of sulfur amino acids (diet AD) than by placing them on a soybean protein sulfur amino acid-deficient

<sup>6</sup> Courtesy of the Pathology Department, University of Pittsburgh.

diet. The rather high liver NPSH value which was obtained for mice kept on diet AD for 7 days indicates that even for these mice a metabolic adjustment had occurred, leading to a partial return of the liver NPSH toward the original level.

From a comparison of the summarized data (table 3) obtained for: (a) mice fed the soybean protein diets, and (b) mice fed the pure amino acid diets, it is apparent that low liver NPSH values were correlated with the use of

TABLE 2

*The effect of a dietary deficiency of sulfur amino acids on non-protein sulfhydryl (NPSH) of mouse liver, with special reference to duration of deficiency*

DAYS ON DIET BEFORE SACRI- FICE	LIVER NPSH, AS MG % GSH EQUIVALENT <sup>1</sup>				% DECREASE IN LIVER NPSH ASSOCIATED WITH USE OF DEFICIENT DIET	
	Protein experiments		Amino acid experiment		Protein exps.	Amino acid exps.
	Adequate diet DA <sub>2</sub> or DA <sub>1</sub>	Deficient diet DD <sub>2</sub> or DD <sub>1</sub>	Adequate diet AA	Deficient diet AD		
1	263 (4)	124 (4)	283	149	53	51
2	277 (2)	184 (2)	269 (3)	114 (3)	34	58
3	237 (2)	142 (2)	277 (4)	106 (4)	40	62
4	279 (2)	172 (2)	331	154	38	53
7	267 (4)	182 (4)	312	211	32	32
14	278 (2)	203 (2)	322	95 <sup>2</sup>	27	70 <sup>2</sup>
21	292 (2)	199 (2)	—	—	32	—

<sup>1</sup> Figure in brackets after liver NPSH value indicates number of separate analyses contributing to value, each analysis being for a single extract prepared from livers pooled from all mice (4 or 5) in a given cage-group.

<sup>2</sup> Mice very apathetic, bedraggled and underweight.

diets low in sulfur amino acids, rather than with food intakes and weight changes exhibited by mice on these particular diets. It is also apparent that the decreases in mouse liver NPSH concentration induced by sulfur amino acid deficiency were far greater than the decreases (if any) induced by this deficiency in the other tissues studied (kidney, spleen and heart).

*Diets unusually rich in sulfur amino acids.* Data obtained in experiments of this type, in which the soybean protein constituted the chief source of dietary nitrogen, are shown

TABLE 3

*Summary of data obtained in experiments using sulfur amino acid-deficient diets*

FACTOR	PROTEIN EXPERIMENTS			PURE AMINO ACID EXPERIMENTS				
	n <sup>1</sup>	Adequate diets DA <sub>2</sub> and DA <sub>3</sub>	Deficient diets DD <sub>2</sub> and DD <sub>1</sub>	p <sup>2</sup>	n <sup>1</sup>	Adequate diet AA	Deficient diet AD	p <sup>2</sup>
Liver NPSH	18	271.2 ± 5.9	173.4 ± 8.6	< 0.001	11	288.1 ± 14.4	122.2 ± 11.4	< 0.001
Kidney NPSH	18	169.4 ± 2.4	164.2 ± 3.1	NS <sup>2</sup>	11	160.8 ± 6.4	136.1 ± 6.2	0.02
Spleen NPSH	17	122.9 ± 2.1	114.8 ± 1.9	0.01	6	118.0 ± 4.7	94.5 ± 4.7	0.03
Heart NPSH	18	43.4 ± 1.3	41.0 ± 1.3	NS	7	41.0 ± 1.4	33.8 ± 2.2	0.03
Food intake (grams) per mouse per day)	18	2.88 ± 0.06	3.27 ± 0.11	0.01 <sup>4</sup>	11	2.15 ± 0.13	1.89 ± 0.14	NS
Wt. change (grams) per mouse per day) <sup>2</sup>	18	+ 1.50 ± 0.049	- 0.073 ± 0.061	0.01 <sup>4</sup>	11	- 1.05 ± 0.16	- 1.49 ± 0.18	NS

<sup>1</sup> Number of comparisons made.

<sup>2</sup> Probability that difference of means was due to chance.

<sup>3</sup> Difference not statistically significant (p greater than 0.05).

<sup>4</sup> In the Drackett protein experiments, sulfur amino acid deficiency resulted in weight loss in spite of an increase in food intake.

<sup>5</sup> Plus values, weight gain; minus values, weight loss.

in table 4. It is apparent from these data that a considerable increase in the content of methionine or L-cystine, or both, over the amounts of these sulfur amino acids provided in control diet DA1, failed to induce a measurable increase in the NPSH concentration of mouse liver, kidney or spleen. In fact, appreciably lower liver NPSH values were found for mice fed the most highly supplemented diet, DX3, than for mice fed the control diet DA1.

Very similar observations were made in another series of experiments lasting one to 4 days, in which tissue NPSH values of mouse liver, kidney, spleen and heart were com-

TABLE 4

*Tissue NPSH not significantly increased by use of diets highly supplemented with sulfur amino acids*

DIET <sup>1</sup>	AV. FOOD INTAKE	AV. WT. CHANGE	AV. TISSUE NPSH VALUES, AS GSH EQUIV., MG % <sup>2</sup>		
			Liver	Spleen	Kidney
DA1	3.2	+ 0.23	306	105	170
DX1	3.0	— 0.17	265	106	182
DX2	3.4	+ 0.16	268	119	166
DX3	2.4	— 0.33 <sup>3</sup>	224 <sup>3</sup>	104	143

<sup>1</sup> For meaning of symbols, see Methods and table 1.

<sup>2</sup> Averages for groups of mice sacrificed at 1, 3, 7 and 14 days after beginning of dietary regime.

<sup>3</sup> Mice appeared increasingly unkempt and bedraggled as experiment continued.

pared for (I) mice fed a control diet containing 20% casein, or (II) a test diet containing 20% casein plus: (a) 1% L-cystine, (b) 5% L-cystine, (c) 1% L-cystine HCl plus 2.5% DL-methionine, or (d) 2% L-cystine HCl plus 5% DL-methionine.

*Variation in sulfur amino acid content of diet on protein sulfhydryl as percentage of extractable liver protein.* Procedures employed in estimating extractable liver protein sulfhydryl, as a percentage of the extractable protein, have been described elsewhere (Beck, Linkenheimer and Marracini, '54). In the present experiments, protein sulfhydryl was found to constitute about 0.2% of the extractable protein.

regardless of the previous dietary status of the mice contributing livers subjected to analysis. It would appear that the sulfhydryl content of extractable mouse liver protein is not easily altered by dietary procedures.

*Combined effects of (A) dietary deficiency in sulfur amino acids and (B) trauma, on liver NPSH values.* Work in this laboratory (Beck and co-workers, '52, '54; Linkenheimer, '54) has established that within a few hours after induction of severe trauma, mice and rats exhibit markedly decreased liver NPSH values. The present experiments have afforded a very simple dietary procedure for bringing about a similar decrease in liver NPSH, without injury.

In an experiment designed to test for combined effects of: (a) sulfur amino acid dietary deficiency, and (b) tourniquet trauma, on mouse liver NPSH, the following average liver NPSH values were obtained: (I) 5 controls, 262 mg%; (II) 4 mice placed on diet AD, devoid of sulfur amino acids, for 28 hours, 124 mg%; (III) 5 mice sacrificed one hour after removal of hind leg ligatures, and 4 hours after ligatures had first been applied, 129 mg%; and (IV) 5 mice subjected to both procedure (II) and procedure (III) above, 142 mg%.

These data indicate that part of the liver NPSH is much more labile than the rest, and that once this labile NPSH has disappeared from the liver, as for example by placing mice on diet AD for 24 hours, it cannot be made to disappear again, as by induction of trauma.

*X-ray experiments.* Pertinent data have been summarized in table 5. It is apparent that the x-radiation-induced mortality rate for mice which had been placed on a sulfur amino acid-deficient diet for a few days, and which control tests indicated had markedly decreased liver NPSH values, was not appreciably or significantly different from the mortality rate induced in mice which had been maintained throughout on diets adequate in their sulfur amino acid content. It should be noted that mice kept on diet AD, completely devoid of sulfur amino acids, possessed spleens less than half

as heavy as those of mice maintained on a diet adequate in its sulfur amino acid content.

*Rat weanling experiments.* Two experiments were performed. In the first the special diet period was 14 days. The liver NPSH values for weanling rats were as follows: control amino acid diet R, 268 mg%; diet without valine, 283 mg%; diet devoid of isoleucine, 207 mg%; and for the diet devoid of threonine, 310 mg%. The same liver NPSH value of 217 mg% was secured for each of the control groups of experiment 2. One control group was fed diet R for three days, the other

TABLE 5

*Effect of short-term sulfur amino acid deficiency on: (a) liver NPSH values, and (b) susceptibility to semi-lethal whole body x-irradiation*

EXP	DIET	DAYS ON DIET PRIOR TO IRRADIATION	ESTIMATED LIVER NPSH AT TIME OF IRRADIATION <sup>1</sup>	ESTIMATED AV. X-RAY DOSE (r)	NO. OF 30-DAY SURVIVORS <sup>c</sup>
					No. of irradi- ated mice
VII	DA1	1	276	560	2/31
	DD4	1	124	560	5/30
X	AA	2	247	504	7/24
	AD	2	106	504	5/24
XII	AA	3	218	504	5/23
	AD	3	85	504	7/23

<sup>1</sup> Data for mice on same diet as x-irradiated mice, but sacrificed shortly before x-irradiation. Each value is for 8 to 10 pooled livers.

for 14 days. The test group fed diet R-S, devoid of sulfur amino acids, for three days gave a liver NPSH value of 78 mg%, that fed diet R-S for 14 days a liver NPSH value of 39 mg%. Only a sulfur amino acid dietary deficiency resulted in marked decrease in liver NPSH. Appreciable to marked weight losses occurred in association with each of the specific amino acid deficiencies, whereas weanlings fed the complete diet, R, showed good weight gains over a 14-day test period.

## DISCUSSION

Data presented in this paper indicate that the mouse possesses intracellular homeostatic mechanisms which are remarkably effective in maintaining total concentrations of nonprotein sulfhydryl compounds of kidney, spleen and heart within rather narrow limits, characteristic for each tissue, in spite of drastic alterations in dietary intake of the sulfur amino acids, cystine, cysteine or methionine or both. It should be noted that each of these amino acids has been demonstrated to be a good source material for incorporation into the naturally occurring cysteine-glutathione group of non-protein sulfhydryl compounds of animal tissues (Umbreit, '52).

In relation to dietary effects on tissue NPSH, liver constitutes a special case. Leaf and Neuberger ('47) have reported that in the rat, dietary supplementation with large amounts of sulfur amino acids resulted in an appreciable increase in liver glutathione, estimated by both specific and non-specific methods, while a dietary deficiency in these amino acids resulted in marked decrease in liver glutathione. We have failed to find an increase in mouse liver NPSH with moderate to high sulfur amino acid dietary supplementation. Mice placed on very highly supplemented diets actually exhibited significantly lower NPSH values than did the corresponding controls, and appeared to be unhealthy. This is not entirely surprising in view of the finding by Earle and Victor ('42) that liver hemorrhage and necrosis are induced by prolonged excessive dietary intake of L-cystine.

A marked decrease in mouse liver NPSH did occur in association with sulfur amino acid dietary deficiency. However, even in relation to mouse liver NPSH, homeostatic mechanisms appeared to be operating, since mice subjected to sulfur amino acid deprivation over a period of several days actually exhibited a considerable return of liver NPSH toward normal values.

It is well established (Patt, '53) that prior administration of large amounts of various sulfhydryl compounds results

in a significant decrease in mortality induced in mice and other species by whole body x-irradiation. Organ shielding experiments (Jacobson, '52) indicate that the spleen and liver are particularly important in relation to naturally existing resistance to lethal effects of whole body x-irradiation. Since in the present experiments mice fed diets low in sulfur amino acids exhibited very low liver non-protein sulfhydryl concentrations, and spleens less than half as large as those exhibited by control mice, it would not have been a matter of surprise if the mice fed the diets low in sulfur amino acids had exhibited increased susceptibility to whole body x-irradiation. Actually no effect of the sulfur amino acid dietary deficiency on susceptibility to whole body x-irradiation was found. Since liver protein sulfhydryl is appreciably greater than liver non-protein sulfhydryl (Beck, Linkenheimer and Bianconi, '54), it is possible that the diet-induced changes in *total* liver sulfhydryl were unimportant in relation to sulfhydryl action against x-ray-induced deaths. It is also possible that sulfhydryl protective action is exerted predominantly at some site other than the liver or spleen or both, or that it is non-specific in nature, and replaced by an adjustment of unknown nature, occurring simultaneously with a diet-induced marked decrease in concentration of liver non-protein sulfhydryl.

#### SUMMARY

The only appreciable change in mouse tissue sulfhydryl concentration found to occur in association with alteration in dietary content of sulfur amino acids was a marked decrease in liver non-protein sulfhydryl, occurring in association with use of diets deficient in sulfur amino acids.

In rat weanlings, a marked decrease in liver non-protein sulfhydryl occurred in association with a dietary lack of sulfur amino acids, but not with a lack of valine, of isoleucine, or of threonine.

Mice placed on sulfur amino acid-deficient diets which were effective in markedly decreasing liver non-protein sulfhydryl.



exhibited susceptibilities to semi-lethal x-irradiation indistinguishable from those exhibited by mice maintained on diets adequate in sulfur amino acid content.

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# THE RELATIONSHIP OF VITAMIN B<sub>6</sub> TO SERUM PROTEIN AND NONPROTEIN NITROGEN IN THE RAT DURING PREGNANCY<sup>1</sup>

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Numerous investigators have reported changes occurring in serum protein levels (Plass and Matthew, '26; Robinson et al., '51; Dieckmann, '52; Macy and Mack, no date) and in nonprotein nitrogen and urea levels (Plass, '24; Stander, '24; McGanity et al., '49; Dieckmann, '52; de Alvarez and Richards, '54) in complicated and uncomplicated human pregnancies. However, there have been no reports on the serum protein values of pregnant rats and there is a paucity of data on serum nonprotein nitrogen values (Parsons, '30). The present study was undertaken to investigate protein and nonprotein nitrogen levels in the serum of the rat during pregnancy. Specifically, we were interested in studying both the effects of depleting maternal vitamin B<sub>6</sub> stores prior to mating and the effects of a pyridoxine deficiency during pregnancy on the concentration of protein and nonprotein nitrogen in the serum. Data collected on maternal nitrogen retentions, liver weight, moisture and nitrogen content, as well as data on the offspring of rats subjected to a pyridoxine deficiency during pregnancy, were reported recently (Ross and Pike, '56).

## EXPERIMENTAL METHOD

Female albino rats of the Sprague-Dawley strain were maintained on laboratory chow<sup>3</sup> until they attained a weight of

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<sup>3</sup> Purina.

approximately 200 gm. When regular estrous cycles were established the animals were randomly divided into two main groups of 50 animals each. The main groups were randomly divided into 5 diet groups containing 10 animals each. Group I, referred to hereinafter as the non-depleted group, was maintained on laboratory chow until the day mating was confirmed; group II, the depleted group, was subjected to a prior depletion period (vitamin B<sub>6</sub>-deficient basal ration plus 0.5 mg% of desoxypyridoxine) for at least 7 days before mating. The length of the depletion period varied from 7 to 30 days because of irregularity of estrous cycles in animals subjected to vitamin B<sub>6</sub> deficiency. A group of animals that could not be bred because of cessation of the estrous cycle after 8 days on the depletion diet was sacrificed after 32 to 60 days of depletion and the serum analyzed as it was for the pregnant animals.

On the morning that mating was confirmed by the presence of sperm in the vaginal smear the animals were placed in individual cages and then received the basal ration to which was added one of the 5 supplements shown in table 1. Except for the prior depletion period, there was no difference in the diets offered the two main groups.

On the 22nd day of pregnancy the animal was sacrificed, the heart exposed and blood taken by heart puncture for immediate serum analyses. Serum was analyzed for total protein by a micro-Kjeldahl method employing direct Nesslerization, and for albumin by a method which involved the separation of the albumin and globulin fractions by precipitation with Na<sub>2</sub>SO<sub>4</sub> (Hawk, Oser and Summerson, '47). Globulin values were determined by difference. Nonprotein nitrogen concentration of the serum was determined by the method of Folin and Wu ('19).

The data were analyzed statistically by means of analysis of variance. Comparisons were made between the two main groups to test for the effects of vitamin B<sub>6</sub> depletion prior to pregnancy, and within each main group to test for the effect of the level of vitamin B<sub>6</sub> during pregnancy. There are

TABLE 1  
Composition of experimental diets

BASEL RATION		SUPPLEMENTS <sup>1</sup>		
	%	mg	Diet	
Casoin, vitamin test <sup>2</sup>	26	2.0		
Sucrose and vitamin mixture	9.85	2.0		
Cornstarch	34	200.0	1	Desoxy- pyridoxine mg %
Hydrogenated fat <sup>3</sup>	19	10.0	2	Pyridoxine mg %
Corn oil <sup>4</sup>	5	8.0	3	
Salt mixture <sup>5</sup>	1	0.04	4	
Agar	2	400.0	5	
L-cystine	0.15	4.0		
Vitamin ADE mixture <sup>6</sup>	+	0.4		
		400.0		
		1.0		

<sup>1</sup> Incorporated into diet.

<sup>2</sup> Made up to 9.85 gm with sucrose.

<sup>3</sup> Labco.

<sup>4</sup> Glisco.

<sup>5</sup> Marola.

<sup>6</sup> Hawk and Over — Science, 74: 369, 1931.

<sup>7</sup> Trituration of 0.1% crystalline vitamin B<sub>12</sub> in mannitol (Morek).

<sup>8</sup> ADE mixed in corn oil contained 5,000 I.U. of A, 400 I.U. of D<sub>3</sub> and 10 mg of alpha tocopherol in two drops and was administered two drops per rat every three days.

data for less than 10 animals in some of the diet groups due to refusal to mate or pseudo-pregnancies. However, all the analyses were corrected for disproportionality among the groups.

#### RESULTS AND DISCUSSION

The results of all the serum analyses are shown in table 2.

The average total protein values per 100 ml of serum were lower on all the diets in the depleted than in the non-depleted group, but there was little difference within the two main groups. Analysis of variance showed that only the differences due to depletion were significant ( $P=0.01$ ). However, the data from the depleted animals that could not be bred indicate that there are no changes in the concentration of total protein of the serum due to vitamin B<sub>6</sub> deficiency per se, and this confirms the report from Beaton's laboratory ('53a). It appears, therefore, that the reduction in the concentration of total protein in the serum observed in the depleted pregnant animals is due to the combined effects of pregnancy and depletion.

The average values for albumin per 100 ml of serum varied little between the depleted and non-depleted groups. Statistically the slight differences in serum albumin content were not significant. The serum albumin values observed for all of the pregnant animals were lower than those for the depleted animals that could not be bred. It appears, therefore, that serum albumin is reduced during reproduction in the rat. Further, it appears from the data obtained that the maintenance of albumin levels during pregnancy is not affected by the vitamin B<sub>6</sub> deficiency imposed under the conditions of this study.

The concentrations of globulin per 100 ml of serum were lower for all the animals in the depleted than for those in the non-depleted group. The lowest values appeared in the depleted animals maintained during gestation on the desoxy-pyridoxine-supplemented and pyridoxine-free rations. Analysis of variance showed that the differences were significant

TABLE 2  
Average protein and nonprotein nitrogen levels of the serum

PYRIDOXINE EXPERIMENT	No. of animals	NON DEPLETED				DEPLETED				No. of depletion days
		Total protein	Albumin	Globulin	NPN	Total protein	Albumin	Globulin	NPN	
		gm %	gm %	gm %	mg %	gm %	gm %	gm %	mg %	
0.1	9	6.31 ± 0.19 <sup>a</sup>	3.09 ± 0.15	3.31 ± 0.21	34.5 ± 2.5	5.80 ± 0.32	3.29 ± 0.17	2.51 ± 0.30	24.4 ± 1.3	12 ± 1
0	10	6.15 ± 0.27	3.22 <sup>a</sup> ± 0.12	3.07 <sup>a</sup> ± 0.20	35.3 ± 2.5	5.40 ± 0.30	3.22 <sup>a</sup> ± 0.24	2.12 <sup>a</sup> ± 0.31	27.3 ± 1.1	14 ± 2
0.1	10	6.15 ± 0.29	3.22 ± 0.12	2.93 ± 0.26	33.8 ± 1.6	5.71 ± 0.18	3.02 <sup>a</sup> ± 0.14	2.72 <sup>a</sup> ± 0.12	31.0 ± 1.7	14 ± 2
0.9	10	6.10 <sup>a</sup> ± 0.23	3.11 <sup>a</sup> ± 0.13	2.99 <sup>a</sup> ± 0.25	34.5 ± 1.4	5.85 ± 0.24	3.46 <sup>a</sup> ± 0.12	2.51 <sup>a</sup> ± 0.26	32.4 ± 1.1	19 ± 3
1.2	10	6.39 ± 0.11	3.56 ± 0.11	2.82 ± 0.23	34.7 ± 2.0	5.81 ± 0.15	3.32 ± 0.10	2.50 ± 0.17	30.0 ± 1.6	16 ± 3
0.5 <sup>b</sup>						6.65 ± 0.35	3.80 ± 0.23	2.85 ± 0.35	34.6 ± 1.3	52 ± 4

<sup>a</sup> Plus 0.5 mg % desoxypyridoxine.

<sup>b</sup> Standard error of the mean.

<sup>c</sup> Average for 9 animals.

<sup>d</sup> Average for 7 animals.

<sup>e</sup> Average for 6 animals.

<sup>f</sup> Average for 8 animals.

<sup>g</sup> These animals could not be bred because of cessation of estrous cycles after 8 days on the depletion diet.

due to the effect of prior depletion ( $P=0.01$ ) but were not significant due to diet. The highest concentration of globulin per 100 ml of serum was observed in the non-depleted animals maintained on the pyridoxine-deficient diets during pregnancy. When the animals were depleted prior to mating, thereby producing what would appear to be a more severe tissue deprivation of vitamin B<sub>6</sub>, there were less marked elevations of serum globulin. The concentration of globulin in the serum of the animals that could not be bred but were subjected to the stress of pyridoxine deficiency was similar to that observed for animals receiving vitamin B<sub>6</sub> and subjected to the stress of pregnancy.

Marked changes in the nonprotein nitrogen in the blood usually have been shown to be due to altered urea levels, since urea constitutes the largest fraction of the nonprotein nitrogen constituents. Although only total nonprotein nitrogen was determined in this study, for the purposes of this discussion it is assumed that any differences in the levels of nonprotein nitrogen were due to variations in urea.

It may be observed that the average nonprotein nitrogen concentrations in the serum of the depleted animals were lower than those for the non-depleted group. The sera of the depleted animals receiving the diet containing desoxypyridoxine had the lowest average nonprotein nitrogen content; as the pyridoxine intake increased, the percentage concentration of nonprotein nitrogen increased. Analysis of variance showed that the differences in nonprotein nitrogen were significant for the effects of depletion on the response to the desoxypyridoxine-supplemented and pyridoxine-free rations ( $P=0.01$ ). There was a significant interaction ( $P=0.05$ ) indicating that the effect of depletion had some influence on the response to these diets. The differences observed between the depleted and non-depleted groups receiving pyridoxine were not significant.

The concentrations of nonprotein nitrogen in the sera in both the non-depleted and depleted groups were lower than those observed by others in non-pregnant animals (Parsons,

'30; Hawkins, MacFarland and McHenry, '46). The depleted animals that could not be bred also had lower nonprotein nitrogen concentrations than those reported for non-pregnant rats, indicating that a vitamin B<sub>6</sub> deficiency also leads to a reduction in nonprotein nitrogen. This is in contrast to reports that an increase in blood urea occurs as a result of a vitamin B<sub>6</sub> deficiency (Beaton et al., '53b; Caldwell and McHenry, '53). The reduction in the nonprotein nitrogen concentrations in the sera observed either as a result of the vitamin B<sub>6</sub> deficiency, or as a result of pregnancy, was intensified by combining the effects of the deficiency and pregnancy. This compound effect was similar to that previously noted for total protein in the serum.

It is of interest that these data on pregnant rats show a trend similar to that reported for humans. The slight decrease in the concentration of total protein which was observed for the pregnant animals is similar to the decrease in total protein reported for uncomplicated human pregnancy (Plass and Matthew, '26; Robinson et al., '51; Dieckman, '52; Macy and Mack, no date). The intensification of the reduction in the concentration of total protein of the serum observed in the depleted animals is similar to what has been reported in complicated human pregnancies (Robinson et al., '51; Macy and Mack, no date). It appears, also, that serum albumin is reduced during pregnancy in the rat as it is in the human (Dodge and Frost, '38; Rinehart, '45). Further, the changes in serum globulin concentration are similar to those observed in human pregnancy: increases in the concentration of serum globulin in uncomplicated pregnancy with peak levels in mild complications (Robinson et al., '51; Macy and Mack, no date) and a tendency to decrease as the severity of the complications progress (Dieckmann, '52). The decrease in the concentration of nonprotein nitrogen in the serum of the pregnant rats is similar to the decrease reported for pregnant women (Plass, '24; Stander, '24). The significant decrease in nonprotein nitrogen levels of the depleted animals is similar to the de-



crease in blood urea values reported by some investigators for toxemic patients (Plass, '24; McGanity et al., '49).

#### SUMMARY AND CONCLUSIONS

The effects of depleting maternal vitamin B<sub>6</sub> stores prior to mating and of a pyridoxine deficiency during pregnancy on the concentrations of serum protein and nonprotein nitrogen in the serum of the rat were investigated.

Depletion of maternal vitamin B<sub>6</sub> stores prior to mating or pyridoxine deficiency during pregnancy or both, lead to changes in serum protein and nonprotein nitrogen concentrations in the rat which are similar to those reported for the toxemias of pregnancy.

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# INFLUENCE OF DIET COMPOSITION ON CALORIC REQUIREMENTS, WATER INTAKE AND ORGAN WEIGHTS OF RATS DURING RESTRICTED FOOD INTAKE<sup>1</sup>

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The literature contains few reports on food intake, water consumption and organ weights of animals on restricted diets. It is well known, of course, that humans on restricted food intake develop adaptive mechanisms. When these were first examined experimentally about 50 to 80 years ago, it was found that starvation leads to reduced energy requirements. In the rat in particular, this problem was studied more recently by Swift and French ('54) and by Quimby ('48). However, interest has centered mainly around questions of energy requirements and basal metabolism and surprisingly little work has been done about other adaptive changes such as those in the growth patterns of individual organs, or about the influence of the main nutrients on the adaptive mechanisms.

If feeding can be restricted just to the point where the weights of the animals are kept constant, there is the advantage that some data can be more specifically compared inasmuch as there are no differences in growth rates or body weights to be considered.

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Thus it seemed to be desirable to study the influence of diets high in protein, fat, or carbohydrate upon food consumption, water intake, and organ weights in rats whose weights had been kept constant.

#### EXPERIMENTAL

The experiments were carried out on male albino rats derived from a homogenous colony. They were delivered by the dealer when they weighed about 50 gm (a little over three weeks of age) and were immediately placed on a purified diet containing 30% lactalbumin, 10% commercial lard, 54% cerelese, 4% salts (USP II), 2% roughage,<sup>3</sup> and liberal amounts of all known vitamins.<sup>4</sup> This diet permitted excellent growth. Eight days after delivery, the rats were earmarked and weighed; 4 days later, they were reweighed. Matching groups of 8 rats each were formed whose average weights at 31 days and again at 35 days were identical. They were now housed in single unit cages with wire bottoms, weighed daily except Sunday, and given the appropriate amounts of food to keep their weights constant. It was possible to maintain the average weight of the rats in each group within 2 gm throughout the periods of examinations, which sometimes lasted 4 months.

Water intake was determined by the difference in weight of the full and partially empty bottles, which had been fitted with machine-made stems permitting no dripping but free drinking.

The composition of the three main diets is given in table 1. The caloric values of the diets were calculated by assuming that carbohydrate and protein yielded 4 and fat, 9.2 Cal. per gram. These values seem to be commonly accepted; but even if somewhat different values, such as those found by Thomson

<sup>3</sup> Alphacel, powdered and extracted rice bran hull, supplied by Nutritional Biochemicals Corp., Cleveland, Ohio.

<sup>4</sup> We are indebted to Dr. Leo A. Pirk of Hoffmann-La Roche, Inc., Nutley, N. J. for most of the synthetic vitamins used in these experiments. Barnett Laboratories, Long Beach, California, kindly supplied us with the crystalline beta-carotene and Dr. M. L. Tainter of the Sterling Winthrop Research Institute of Rensselaer, N. Y., gave us the crystalline vitamin D<sub>3</sub>.

and Munro ('55) were preferable, our conclusions would not be altered. The cellulose content of the diets has been disregarded in calculating their caloric value because, even in the unlikely case that the rats utilized 10% of the cellulose in the diet containing 11% of this material, the caloric value of the diet would have been increased by less than 1%.

In comparing the utilization of the various diets, it was convenient to calculate the calories needed by the rat for the maintenance of 1 gm of body weight during one week. Earlier studies had shown that the rat needs 1 gm of a "normal" diet over and beyond that required for weight maintenance to gain 1 gm of body weight. With this in mind, it was pos-

TABLE 1  
*Composition of experimental diets<sup>1</sup>*

DIET	CASEIN	CERELOSE	LARD	CELLULOSE	SALTS
	%	%	%	%	%
High fat	20	0	65	11	4
High protein	70	0	20	6	4
			None (2% Lino- leic acid)		
High carbohydrate	15	75		4	4

<sup>1</sup> Plus all accessory food factors.

sible to correct the weekly food intake for minor changes in the body weight. Thus, the total weekly individual food intake was increased or decreased by 1 gm for each gram of body weight lost or gained by the rat during the week. This gram for gram correction was used only in the case of the high-protein and high-carbohydrate diets. For the rats on the high-fat diet, the food intake was modified by 0.5 gm for each gram of change in body weight. The corrected weekly individual food intakes of each group were averaged, divided by the average weight of the group, and converted to calories. This value of calories per gram body weight per week was used for comparing the caloric requirements of the various groups. Because the requirements for weight main-

tenance are sensitive to changes in room temperature, comparisons were made only of groups running simultaneously.

#### RESULTS AND DISCUSSION

When the caloric requirements for weight maintenance were studied on rats given a purified diet including 30% casein, 10% lard, and 54% cerelose for a period of 4 months,, they were found to decline from 1.9 Cal./gm body wt./week to about 1.0 Cal. within the first 5 weeks and to remain fairly constant thereafter. This was in complete agreement with Quimby's findings.

In figure 1 are shown the average caloric requirements and the individual water intakes of groups of rats maintained on high-fat, high-carbohydrate, and high-protein diets. The caloric requirements of the animals on the high-fat and high-carbohydrate diets were initially similar; those for the high-protein diet were lower. The requirements of all groups declined but those for the high-fat diet, more rapidly, so that they eventually were equal to the requirements for high protein. The comparatively lower utilization of carbohydrates by animals on restricted food intake was also suggested in the studies of Rice et al. ('56).

The outcome was not related to the variations in protein intake because, in absolute amounts, the animals on the high-carbohydrate diet consumed only a fraction of that eaten by the rats on high-protein but twice as much as that consumed by the animals on the high-fat regimen.

It may be worth while to emphasize that the depression of the caloric requirements was given only with the fresh fat. We have previously demonstrated that the inclusion of autoxidized fats or their fractions prevents the decline of the caloric requirements during a period of restricted food intake (Kaunitz et al., '56).

The differences in caloric requirements brought on by high-fat, protein, or carbohydrate diets went hand in hand with characteristic differences in water intake shown in figure 1. The weekly intake in milliliters is plotted on a logarithmic

scale, which, for the eye, minimizes differences. However, it is obvious that there existed marked differences among the three groups. The animals on the high-fat diet consumed least, on the average, 45 ml weekly in the earlier weeks and later only 36 ml. Next are the high-carbohydrate animals

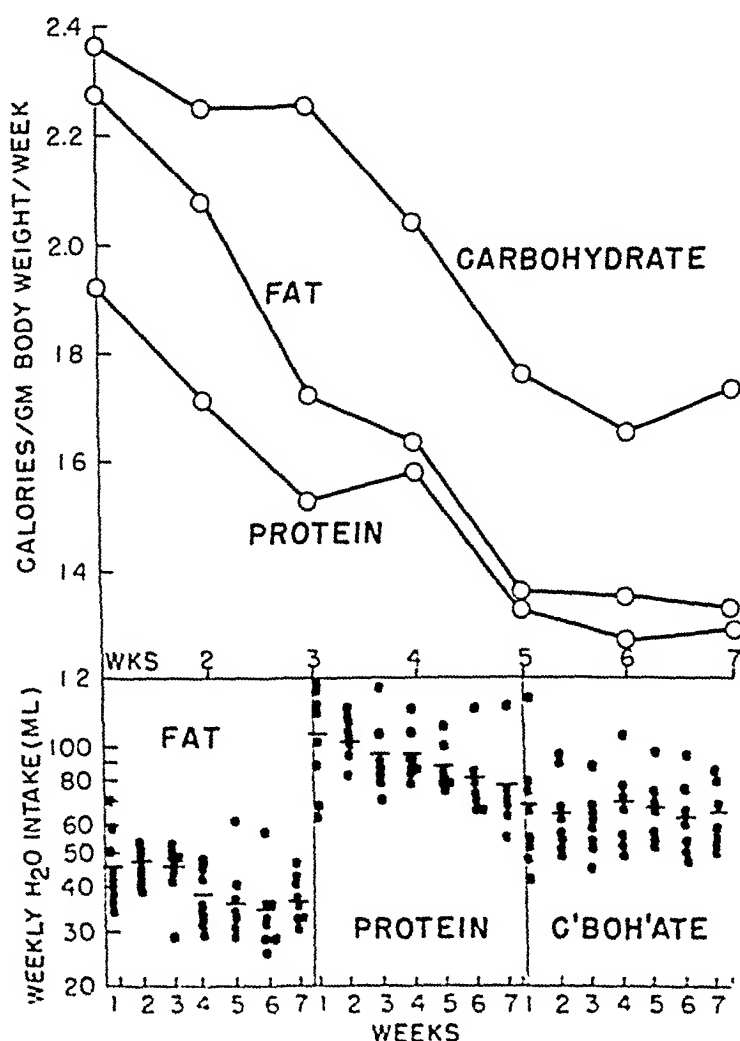


Fig. 1. Average weekly caloric requirements for weight maintenance and weekly individual water intakes of groups of 8 male rats kept at constant weight on diets high in fat, protein, or carbohydrates — represent average for the group.



with average intakes of 65 ml throughout the whole period. The high-protein group had the highest consumption, about 105 ml in the earlier and 75 to 85 ml in the later weeks.

It has been known that protein intake is one of the determining factors for thirst; and, in these experiments, the animals on high-fat diets, having the lowest protein intake, had the lowest water intake and the animals on the high-protein diet drank most. However, the water intake was not linearly related to the protein intake. The animals on high protein ate at least 5 times the amount of protein consumed by those on the high-fat diet. Yet the water intakes differed by a factor of only two and one-half. Furthermore, the substantial decrease in protein intake of all groups during the time of restricted feeding on account of the decrease in caloric requirements was accompanied by only slight decreases in the water intakes of the high-fat and high-protein groups. The water intake of the high-carbohydrate group remained constant throughout and was only slightly below that of the high-protein group although the protein intakes of the two groups varied by a factor of 3.

For this reason, one must assume that the variations in water intake are not only caused by the differences in protein consumption but are also related to the main constituent of the diet. One wonders whether the effect of fat in reducing thirst has clinically found sufficient attention in conditions where a low water intake is desirable.

When the studies of food consumption and water intake were terminated, the animals were immediately sacrificed and their organs weighed. In table 2 are presented organ weight data calculated per 100 gm of body weight. Such a calculation has some degree of justification in view of the narrow range of the body weights. However, the organ weights were also plotted against the body weights on a log—log scale and the results compared with data for animals which had been allowed to eat freely of a purified control diet. Inferences were drawn only when both methods indicated significant differences.

Liver weights of all groups tended to be low or even sub-normal, a consequence of food restriction. As was to be expected, the kidneys of the animals fed the high-protein diet were heaviest and differed significantly from those of the

TABLE 2

*Organ weights, per 100 gm of body weight, of male rats kept at constant weights by restricted feeding of diets high in fat, protein or carbohydrate<sup>1</sup>*

ORGAN	HIGH FAT 24 animals av. wt. 94 gm range 74-108 gm $\sigma = 8.0$			HIGH PROTEIN 15 animals av. wt. 91 gm range 75-108 gm $\sigma = 9.0$			HIGH CARBOHYDRATE 16 animals av. wt. 100 gm range 90-110 gm $\sigma = 5.0$		
	Av. Wt.	Range	SE	Av. Wt.	Range	SE	Av. Wt.	Range	SE
	gm	gm	gm	gm	gm	gm	gm	gm	gm
Liver (normal range, 3.6-5.4 gm)	3.6	2.9-4.3	0.08	3.8	2.9-5.3	0.15	3.6	2.9-4.2	0.11
Kidneys (normal range, 0.75-1.2 gm)	1.0	0.6-1.3	0.03	1.2	0.9-1.5	0.03	0.9	0.7-1.0	0.03
Adrenals (normal range, 9-27 mg)	27	15-36	1.0	28	17-36	1.4	22	15-28	0.9
Testes (normal range, 0.6-1.4 gm)	1.1	0.5-2.2	0.10	1.1	0.2-2.0	0.16	0.9	0.5-1.5	0.06
Thymus (normal range, 240-550 mg)	80	35-107	5.0	93	60-134	10.0	77	58-105	3.7

<sup>1</sup> "Normal" refers to male rats of 100 gm which had eaten a highly purified control diet ad libitum. The standard deviation,  $\sigma = \sqrt{\frac{\sum d^2}{n-1}}$  where  $\sum d^2$  denotes the sum of the squares of the differences of the individual values from the mean and  $n$ , the number of values. The standard error,  $SE = \frac{\sigma}{\sqrt{n}}$ .

animals fed the high-carbohydrate diet. The adrenals of the animals on the high-fat diet were significantly heavier than those of the animals on the high-carbohydrate diet.

The testicular weights of the high-fat animals were, in more than half the cases, above the upper limit of the normal

spread and differed significantly from those of the other two groups. Thus, on the high-fat diet, the testes had increased in size although the body weight had remained constant. The thymus weights of all animals were considerably below normal, again an expected consequence of food restriction.

#### SUMMARY

1. The influence of high-fat, high-carbohydrate, and high-protein diets on the caloric requirements, water intake, and organ weights of rats kept at constant weight by restricted feeding was studied.

2. The caloric requirements for all diets declined during the first 5 weeks and became constant thereafter. On the high-protein and high-fat diets, the animals were eventually able to maintain their weight with 25% fewer calories than those on the high-carbohydrate diet.

3. The water intake was highest on the high-protein and lowest on the high-fat diet.

4. The adrenal weights of the animals on the high-fat diet were higher, on the average, than those of the animals on the high-carbohydrate diet, with those of the high-protein rats being in between. The renal weights were highest among the high-protein animals. The testicular weight of the high-fat animals was significantly higher than that of the animals on the high-carbohydrate diet.

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## ASCORBIC ACID UTILIZATION BY WOMEN

### RESPONSE OF BLOOD SERUM AND WHITE CELLS TO INCREASING LEVELS OF INTAKE IN TWO GROUPS OF WOMEN OF DIFFERENT AGE LEVELS<sup>1</sup>

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The response of blood serum and white cells to changes in ascorbic acid intake has been reviewed by Steele et al. ('55) and Morse et al. ('56). Steele et al. ('55) found that the ascorbic acid content of the white cells and serum increased significantly when 40 mg of ascorbic acid per day were given for 7 to 11 days, following an intake of 30 mg for 11 to 14 days. Morse et al. ('56) reported that the average white cell levels paralleled the rise in serum levels in a group of 19 women subjects when the intake of ascorbic acid was increased from 33 mg per day to 58 mg and then to 83 mg per day. Correlation between serum and white cells was statistically significant at these levels of intake. An intake of 133 mg per day caused no further increase in average white cell levels, but did produce a significant rise in average serum levels.

The present study is concerned with the relationship between the ascorbic acid level in blood serum and white cells

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<sup>2</sup>Credit is due Dr. Geoffrey Beall, Professor of Statistics, and Dr. Mary L. Greenwood, Associate Professor of Foods and Nutrition, University of Connecticut, for verification of the statistical treatment of the data.

with subjects receiving smaller graded levels of supplementation than in the previous study. Two groups of women of different age levels are compared.

#### PROCEDURE

*Subjects.* Two groups of women, patients at a state training school and hospital for the handicapped,<sup>3</sup> served as subjects during the winter of 1954 to '55. Fifteen had an average age of 31 years (range 28 to 34) and 13 had an average age of 64 years (range 56 to 77). All were in good physical health, and were mentally capable of cooperating in the study. The subjects were examined at the beginning and end of the study for clinical signs of vitamin C deficiency.

*Dietary ascorbic acid.* Throughout the 4 months of the study, the subjects were on the regular institution diet except that all foods high in ascorbic acid, such as citrus fruits, tomatoes, pineapple, and raw cabbage were omitted. This provided a somewhat restricted and relatively constant level of dietary ascorbic acid. Other fruits and vegetables were always served to the subjects in place of those omitted.

The food intake of each subject was recorded on 16 scattered days near the beginning of the study. Food consumption was recorded in terms of the number of servings, or fraction of serving, of each food. Servings were weighed at intervals to determine size of portions. On 44 days, scattered throughout the period, samples of food were collected in the dining hall for analysis for total ascorbic acid by an adapta-

<sup>3</sup> Mansfield State Training School and Hospital, Mansfield Depot, Connecticut. Thanks are extended to the medical and dietary staffs of the school as follows: To Dr. Gail F. Moxon, M.D., and Dr. Harriet Bixby, M.D., resident doctors, for the physical examinations; to Dr. Joseph E. Nowrey, M.D., resident doctor, and his assistants for taking the venous blood samples; to Dr. Luke Grotano, D.D.S., resident dentist, for the dental examinations; to Mrs. Pauline Duckett, chief dietitian, and to the dietary staff of the women's dining room, for cooperation in the collection of dietary data and of food samples; to the 28 mentally retarded women who served so cheerfully and cooperatively as subjects; and to Dr. Neil A. Dayton, M.D., Superintendent of the Training School, for making the institution available for the study and for his continued interest and encouragement in research work.

tion of the 2,4-dinitrophenylhydrazine method of Roe and Kuether ('43). The collection days were determined by the nature of the institution menu. Dietary ascorbic acid intake of each subject was calculated using the food values obtained by analysis. Calories, protein, fat, carbohydrate, minerals and other vitamins were calculated using the U.S.D.A. Agriculture Handbook no. 8 (Watt and Merrill, '50). The average intakes of these nutrients met the recommendations of the National Research Council by 100% with the exception of iron which was 96% of the recommended allowance.

*Ascorbic acid supplementation.* After 7 weeks on the diet restricted in ascorbic acid, the subjects were given daily ascorbic acid supplements, beginning with 15 mg and increasing every 14 days to 25, 40, 50 and 75 mg, respectively.

*Serum and white cell determinations.* Serum and white cell ascorbic acid determinations were made on venous blood samples at the end of the 7-week adjustment period without vitamin C supplementation, and at the end of each two-week test period on the 5 levels of supplementation. The blood samples were always taken at 10:00 A.M., three to 4 hours after an ascorbic acid-free breakfast. Preparation and analysis of the serum samples were carried out according to the procedure outlined in the Northeast Regional Publication on Techniques ('51). The blood samples for white cell determinations were prepared in quadruplicate and the determinations made by the method of Bessey (Gyorgy, '50).

#### RESULTS

*Ascorbic acid intake.* The average daily ascorbic acid intake from food was 32 mg for all the women, based on the 16 days on which food intake was recorded. This average daily dietary intake, plus the vitamin supplementation at the 5 levels, brought the average total daily ascorbic acid intakes for the 5 supplemental periods to 47, 57, 72, 82 and 107 mg respectively. This increased intake produced no noticeable changes in the mild symptoms, exhibited by a few subjects, which might have been interpreted as due to vitamin C deficiency.

*Ascorbic acid levels in serum and white cells.* The young women had an average serum ascorbic acid level of  $0.33 \pm 0.04$  mg per 100 ml of serum at the end of the unsupplemented period of 7 weeks. The older women had a slightly lower level of  $0.24 \pm 0.03$  mg at the end of the unsupplemented period.

The young women had an average white cell ascorbic acid level of  $25.6 \pm 1.16$  mg per 100 gm of white cells after the period on the low vitamin C intake, while the older group had an average white cell level of  $22.2 \pm 2.31$  mg at the same time.

The results of supplementing the dietary intake for 5 two-week periods are given in table 1 and presented graphically in figure 1.

TABLE 1

*Average ascorbic acid intakes, serum levels, and white cell levels.*

Total intake	SERUM LEVELS		WHITE CELL LEVELS	
	Young women	Older women	Young women	Older women
mg/day	mg/100 ml	mg/100 ml	mg/100 gm	mg/100 gm
32	$0.33 \pm 0.04$ <sup>1</sup>	$0.24 \pm 0.03$	$25.6 \pm 1.16$	$22.2 \pm 2.31$
47	$0.57 \pm 0.04$	$0.45 \pm 0.04$	$24.4 \pm 1.21$	$23.2 \pm 1.38$
57	$0.60 \pm 0.04$	$0.54 \pm 0.05$	$35.2 \pm 1.80$	$29.5 \pm 1.70$
72	$0.89 \pm 0.07$	$0.84 \pm 0.07$	$35.3 \pm 1.46$	$34.9 \pm 2.80$
82	$1.54 \pm 0.08$	$1.16 \pm 0.09$	$33.3 \pm 1.33$	$34.6 \pm 2.01$
107	$1.76 \pm 0.07$	$1.42 \pm 0.10$	$32.8 \pm 0.80$	$34.7 \pm 2.14$

<sup>1</sup> Mean  $\pm$  standard error.

## DISCUSSION

The average rise in serum ascorbic acid for the group of young women was steady and was significant with every increasing level of intake except on the second level, namely, the 57-mg intake (see fig. 1). At this level of intake the white cell ascorbic acid rose significantly to its peak for the entire period, namely, about 35 mg per 100 gm of white cells.

Individual responses to increasing levels of intake were reflected in the serum of all of the young women on the first level of intake of 47 mg following a 15-mg supplementation. With the increase of an additional 10 mg, making a total of 57 mg, the white cell levels of all but one of the young women rose, while only 9 showed a rise of serum levels. The white

cell level of one woman had already reached its peak of 34 mg on the 47-mg intake. On the next three levels of supplementation all but one or two subjects showed a rise in serum level at each change in intake.

The average rise in serum ascorbic acid in the older women was steady and significant in each supplemental period. The average white cell ascorbic acid paralleled the serum ascorbic

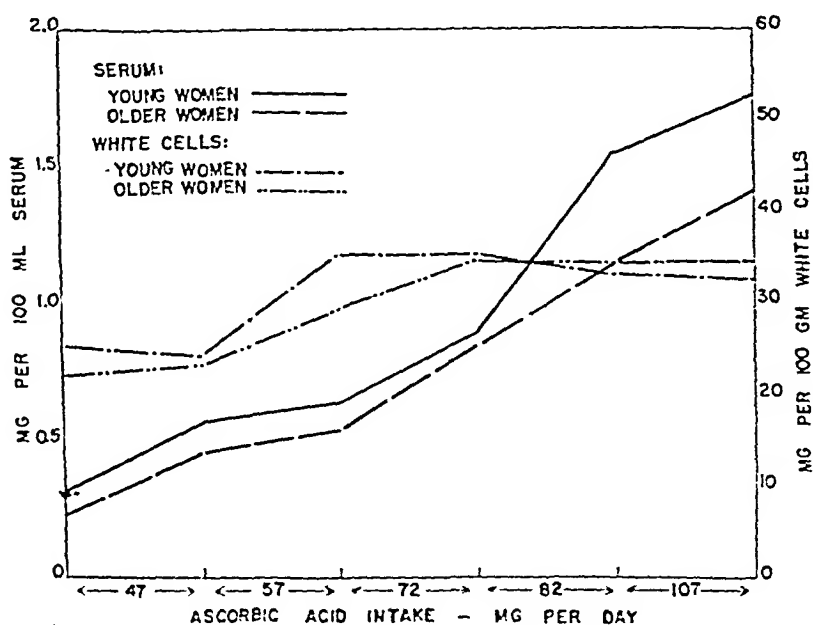


Fig. 1 Average values for ascorbic acid in blood serum and in white blood cells for 15 women near 31 years of age and 13 women near 64 years of age, on a dietary intake of 32 mg, with daily supplements of 15, 25, 40, 50, and 75 mg, each for a two-week period.

acid more closely than in the young group. It reached its peak of about 35 mg for the group on the 72-mg intake, as compared with the 35 mg on the 57-mg intake for the young women.

In the older group, individual responses were similar to those in the young group for serum ascorbic acid. All showed a rise on the 47-mg intake and on the 72-mg intake; all but



two rose on the other levels. Increase in white cell ascorbic acid was found in all but one subject on the 57-mg intake, and in all but three on the 72-mg intake when the average high point was reached.

The above discussion indicates that, at low levels of intake, small increases, such as 10 or 15 mg, resulted in a rise in serum ascorbic acid in the majority of instances. In this study, it seemed to be necessary to go above the level of 47 mg to get a significant average rise in white cell ascorbic acid. In the study by Steele et al. ('55) a significant rise in white cell ascorbic acid was found on increasing the intake from 30 to 40 mg. Their subjects had been maintained at a much lower level of intake previous to supplementation and for a longer period of time than the subjects in this investigation. The smaller increases in levels of intake of ascorbic acid in the present study showed that saturation of white cells may take place on a lower intake level than was exhibited in the earlier study, in which the peak was reached after an intake of 83 mg per day (Morse et al., '56).

Since the average white cell level of the young group reached its peak (35.2 mg) on the 57-mg intake, two weeks earlier than the older group, this might indicate that age had some influence on the rate of uptake of ascorbic acid by white cells. However, no significant difference due to age was noted when a *t* test was applied to the following data:

AVERAGE DAILY ASCORBIC ACID INTAKE	AVERAGE WHITE CELL LEVELS <sup>1</sup>	
	Young group	Older group
57 mg	34.8	29.0
72 mg	35.3	33.6
	$\bar{D}_2 = 0.518$	$\bar{D}_1 = 4.628$

The question is whether  $\bar{D}_1$  is really greater than  $\bar{D}_2$ . It is not significantly so since  $0.05 < P < 0.10$ , or *P* lies between 0.05 and 0.10.

<sup>1</sup> These averages differ slightly from the average values given in table 1 because of using only complete pairs in calculating  $\bar{D}$ .

In contrast to the earlier study of serum and white cell ascorbic acid levels which showed significant correlation at the 33, 58, and 83-mg levels of intake in the group of 19 women (Morse et. al., '56), there was significant correlation in only two instances in the present study. The group of 15 young women showed correlation significant at the 5% level between serum and white cell ascorbic acid on the intake of 32 mg ( $r=0.60$ ) and also on the intake of 47 mg ( $r=0.60$ ). The smaller increases in level of supplementation used in this study and the smaller number of subjects may account for the lack of correlation in the other instances.

For the young group, the regression of white cell level,  $Y$ , on serum level,  $X$ , for the 86 pairs of determinations is:

$$Y = 26.56 + 4.83 X$$

For the older group, the regression of white cell level,  $Y$ , on serum,  $X$ , for the 75 pairs of determinations is:

$$Y = 21.88 + 10.26 X$$

The above equations for the separate groups of women show that the older group experienced a more pronounced rise in white cell ascorbic acid with respect to serum level than the young group, i.e., for every milligram rise in serum the white cells rose 10.26 mg. The regression for the two groups combined as one group is:

$$Y = 24.52 + 6.90 X$$

A test for possible difference in regression between the two groups was made by the method of residual squares. This showed that age difference was not significant since the  $F$  value lies between 0.05 and 0.10.

#### SUMMARY

Following 7 weeks on a 32-mg intake of ascorbic acid, the average serum ascorbic acid level of 15 young women was 0.33 mg per 100 ml. It rose to 1.76 mg during a period of 10 weeks in which the ascorbic acid intake was increased gradually to 107 mg per day. The average white cell ascorbic acid

rose from 25.6 mg per 100 gm of white cells to 35.2 mg during the first 4 weeks when the intake had reached 57 mg per day, and thereafter remained stationary.

The average serum ascorbic acid level for 13 older women, on the same levels of intake, rose from 0.24 to 1.42 mg during the 10-week period. Their average white cell ascorbic acid rose from 22.2 to 34.9 mg during the first 6 weeks when the intake had reached 72 mg per day, and thereafter remained stationary.

Correlation between serum and white cell ascorbic acid levels was significant only in the young group on intakes of 32 mg and 47 mg of ascorbic acid per day. There was no significant difference in uptake of ascorbic acid by the white cells in the young group as compared with that of the older group. Difference in regression of white cell level on serum level between the two groups was not significant.

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# NUTRITIONAL PROPERTIES OF THE MOLECULARLY DISTILLED FRACTIONS OF AUTOXIDIZED FATS<sup>1</sup>

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In previous nutritional work on autoxidized fats, special attention was paid either to the polymer fraction left as a residue after molecular distillation (Kaunitz et al., '55) or to the whole autoxidized fat. It was found that the effects of the polymer fraction or of the whole autoxidized fat could be counteracted by the addition of fresh fat to the diet (Kaunitz et al., '55) and that the caloric requirement of the rat for weight maintenance was increased when such fats were consumed.

The distillate fraction of autoxidized fats, obtained by molecular distillation, had previously been studied only briefly and had not seemed to be particularly remarkable. The further studies to be reported below, however, show that this fraction is also of interest nutritionally.

## EXPERIMENTAL

The studies were carried out on albino rats of a homogeneous colony. Weanling males, when they weighed 40 to 50 gm.

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were placed on a purified diet containing 30% of lactalbumin and 10% of fresh lard. At the age of 5 weeks, they were distributed into matching groups using procedures reported before (Kaunitz et al., '54).

Commercial lard and refined cottonseed oil were aerated at 95°C. for 200 to 300 hours and then distilled, using alembic distillation for the removal of volatile products, followed by molecular distillation. For the latter, temperatures up to 280°C. were employed. In one instance, a hydrogenated cottonseed oil which had been used for deep fat frying for 80 hours at 190°C. was distilled.

Unless otherwise stated, the experimental diets contained 30% alcohol-extracted casein, 10% fat, 54% dextrose, 4% salts (U.S.P. no. 2), and 2% cellulose, as well as liberal amounts of all known accessory food factors<sup>4</sup> in amounts described before (Kaunitz et al., '54).

#### RESULTS AND DISCUSSION

In figure 1 are given the average growth curves of groups of 8 male rats which had been maintained on diets containing various fats. The logarithm of the weight in grams is plotted against the reciprocal value of the age; the advantages of this method have been pointed out by Zucker and Zucker ('42).

The animals receiving autoxidized cottonseed oil lost weight rapidly and died after two to 4 weeks. When 10% of fresh fat was added to the diet containing 10% of the oxidized oil, none of the animals died during the period of observation; they were even able to grow. This has previously been described as the protective effect of fresh fat. One group of animals received 10% of the distillate from the molecular distillation of the sample of hydrogenated cottonseed oil which had been used for deep fat frying. These animals grew essentially as well as did those on fresh cottonseed oil. How-

<sup>4</sup> Doctor Leo A. Pirk of Hoffmann-La Roche, Inc., Nutley, New Jersey, very kindly supplied us with most of the synthetic vitamins used. Vitamin D<sub>2</sub> was supplied by the Sterling-Winthrop Research Institute, Rensselaer, N. Y., and the crystalline beta-carotene, by the Barnett Laboratories, Long Beach, California.

ever, when the distillate was combined with oxidized cottonseed oil, growth was significantly below that of the animals receiving both fresh and oxidized cottonseed oils. Also, in contrast to the latter group, some of the animals died toward

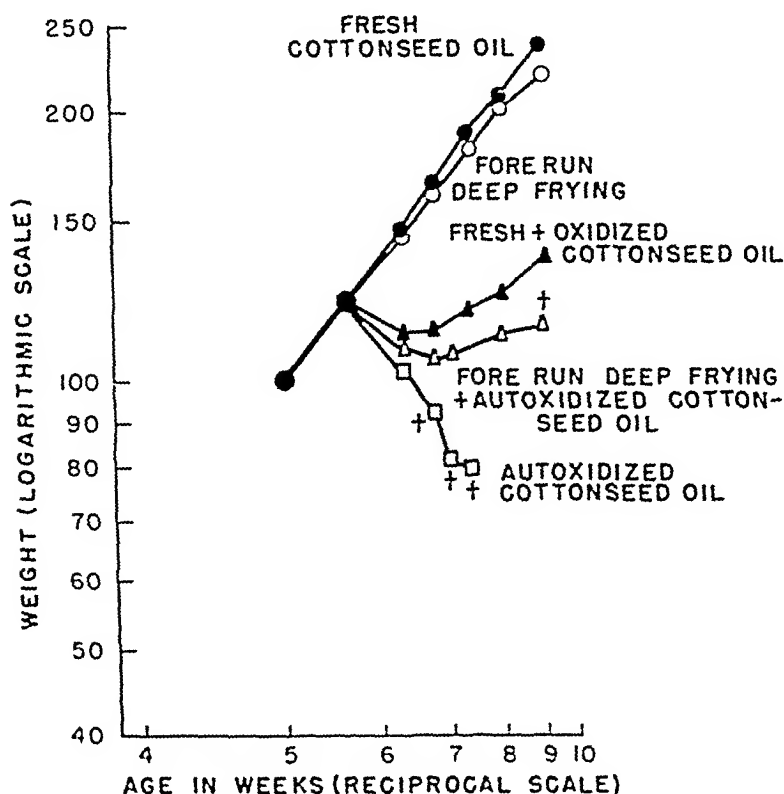


Fig. 1 Influence of the distillate from the molecular distillation of a hydrogenated vegetable oil after its use for deep frying for 50 hours. Each curve is based on the average of 8 well-matched male rats. After the third week of the experiment, the difference in weight of the groups fed oxidized plus fresh fat and oxidized plus distillate is statistically significant.

the end of the period of observation. Therefore, the distillate, while permitting nearly normal growth when included in the diet as the only fat, had lost a high degree of its protective effect.

Six very similar experiments were carried out with the molecular distillation fractions of highly autoxidized cotton-

seed oil or highly autoxidized lard. These distillates usually permitted good, although not quite optimum, growth when used as the sole fat source. Significantly, all of the distillates had lost their protective effect against highly autoxidized cottonseed oil to a degree very similar to that shown in figure 1.

This loss of protective effect could not have been caused by the molecular distillation process. The undistilled autoxidized cottonseed oil containing 40% polymeric "residue" and 60% "distillate" fraction led to rapid deterioration of the animals, whereas a mixture of 40% polymeric residue and 60% fresh oil permitted acceptable growth. Thus, the lack of protective action of the distillate was discernible before the oil had undergone the heating necessary for molecular distillation.

When rats, by daily weighing and restricted feeding, are maintained at a weight constant within 3 gm, it has been observed that the caloric requirements for such weight maintenance decline rapidly within the first few weeks if "good" diets are used (Quimby, '48). It has been shown (Kaunitz et al., '56) that the caloric requirements for weight maintenance do not decrease when the residue fraction of a molecularly distilled autoxidized fat is included in the diet. In figure 2 is shown a similar experiment with fresh fat and the molecular distillate of the hydrogenated vegetable oil which had been used for deep frying. The requirements are expressed as weekly calories per gram of body weight and are the average values for each group of 8 animals. For the calculation of the caloric values of the diets, it was assumed that a factor of 9.2 Cal. per gram could be used for both fats. It seemed reasonable to assume that the caloric value of the distillate did not differ greatly from that of normal fat because, when the distillate was included in a diet as the only fat source and the animals were permitted to eat freely, (1) the resulting growth was only slightly below that of animals fed fresh fat and (2) the food intakes were similar. However, even if

the caloric value of the distillate is slightly below that of the fresh lard, this would not lead to different conclusions.

As can be seen from figure 2, the caloric requirements of both groups declined steeply during the first 4 weeks of observation. The net energy value of the diet containing distillate was lower than that of the diet containing fresh fat, although the difference was not as pronounced as that between the groups fed polymeric residue and fresh fat. However, it

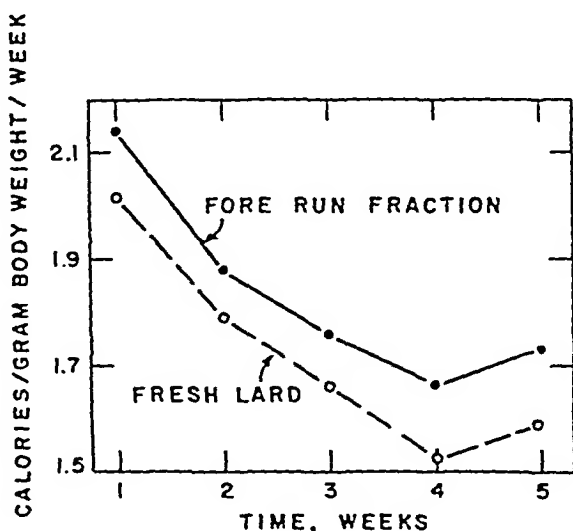


Fig. 2 Influence of fresh lard and the distillate from the molecular distillation of a hydrogenated vegetable oil previously used for deep frying for 80 hours upon the caloric requirements of matching male rats maintained at constant weight. Each curve is based on the average of 8 animals.

may be of some interest that, with a chemically altered but essentially atoxic fat, the animal's caloric requirement for weight maintenance is increased.

When the animals maintained at constant weight were sacrificed at the end of the experiment, their kidneys, livers, and adrenals were weighed. Figure 3 shows log—log plots of organ weights against body weights. The parallel lines give the limits of the spread in organ weight of male rats fed a complete, unrestricted diet. The weights of the livers and



kidneys of the animals on the distillate were within normal limits, although somewhat above those of the animals fed fresh fat. The adrenals of the two groups scarcely differed from one another. In contrast, the livers, kidneys, and adrenals of the animals given the residue fraction substantially

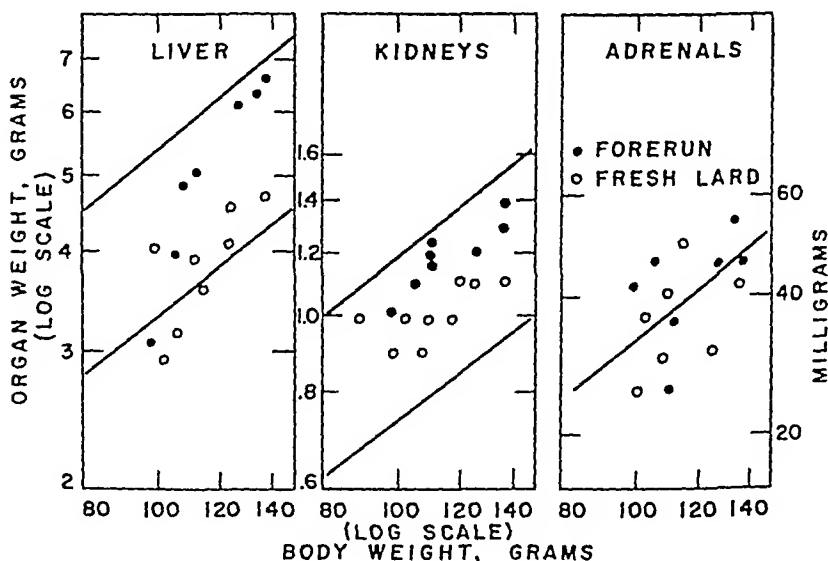


Fig. 3 Influence of fresh lard and distillate from the molecular distillation of a hydrogenated vegetable oil previously used for deep frying for 80 hours upon the organ weight-body weight relationships of male rats kept at constant weight by restricted feeding for 5 weeks. On the log—log plot, the parallel lines indicate the upper and lower limits of the spread in organ weight of rats with unrestricted intakes of a control diet containing fresh lard.

exceeded the upper limit of the normal (Kaunitz et al., '56). These results also show that the distillate itself is hardly toxic.

This low toxicity again became evident in studies with low-protein diets. In earlier work (Kaunitz, '53), it was pointed out that weanling rats placed on diets containing only 5% of casein and fresh fat maintained their weight for several weeks and grew slowly thereafter. Ten per cent of a sample of oxi-

dized lard which was atoxic to rats when included in a diet containing 30% of casein led to rapid weight loss and death when fed in a diet containing only 5% of casein. When 10% of the distillate was included in a diet with 5% of casein, growth of the rats was similar to that of the controls receiving fresh fat.

The chemical changes in the fats responsible for the described effects are not as yet understood. This problem is being actively investigated.

#### SUMMARY

1. Lard and refined cottonseed oil which had been aerated at 95°C. for 200 to 300 hours and a sample of hydrogenated vegetable oil which had been used commercially for deep fat frying for 80 hours at 190°C. were molecularly distilled at 280°C. The distillates were used in nutritional experiments.

2. When the distillates were included in purified diets containing either 5 or 30% casein, the resulting growth of most of the weanling male rats fed these diets was only slightly below that of matching rats receiving fresh lard.

3. In contrast, distillate added to the nonvolatile polymeric residue from the molecular distillation of autoxidized fats had a protective effect markedly below that of fresh fats.

4. The net energy value of the diet containing distillate was lower than that of the diet containing fresh fat.

5. Liver, kidney and adrenal weights of rats fed distillate were within the normal spread for these organs and were only slightly higher than those of the controls, thereby supplying additional evidence for the low toxicity, if any, of these fractions.

#### ACKNOWLEDGMENTS

Doctor Waldo C. Ault of the eastern Regional Laboratory of the U. S. Department of Agriculture has greatly helped this work with his advice, suggestions, and criticisms.

kidneys of the animals on the distillate were within normal limits, although somewhat above those of the animals fed fresh fat. The adrenals of the two groups scarcely differed from one another. In contrast, the livers, kidneys, and adrenals of the animals given the residue fraction substantially

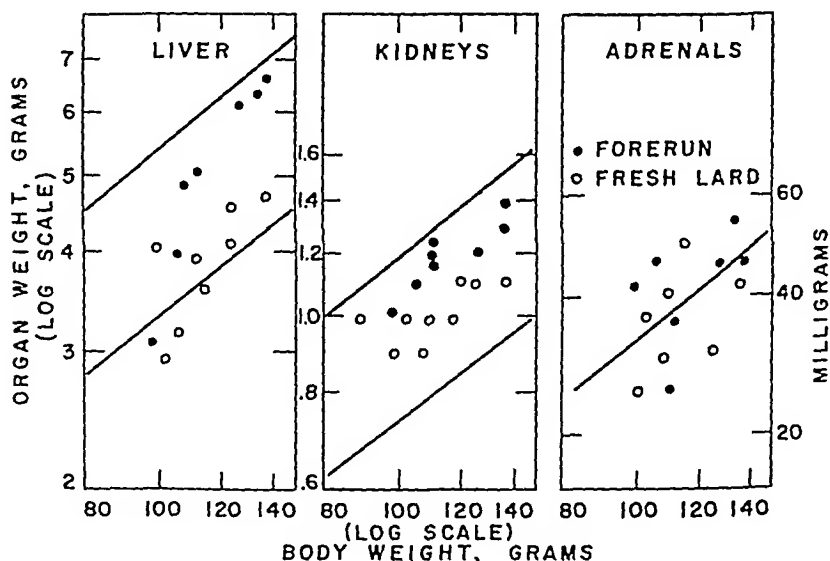


Fig. 3 Influence of fresh lard and distillate from the molecular distillation of a hydrogenated vegetable oil previously used for deep frying for 80 hours upon the organ weight-body weight relationships of male rats kept at constant weight by restricted feeding for 5 weeks. On the log—log plot, the parallel lines indicate the upper and lower limits of the spread in organ weight of rats with unrestricted intakes of a control diet containing fresh lard.

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SOME FACTORS  
AFFECTING CELLULOSE DIGESTION BY  
RUMEN MICROORGANISMS  
*IN VITRO*

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A previous report from this laboratory revealed that cellulose digestion by rumen microorganisms *in vitro* was markedly stimulated by such fishery by-products as whale solubles, herring solubles and herring stickwater as well as by a mixture of 18 amino acids (MacLeod and Brumwell, '54). Although much of the effect of the fishery by-products could be ascribed to their amino acid content, some evidence was obtained that other unknown factors might also be present and capable of stimulating cellulose digestion. The present study was undertaken to investigate this possibility further.

In the previous study a marked stimulation of cellulose digestion was obtained with the various supplements tested only when a more dilute inoculum of rumen liquid than it had previously been the custom to use (cf. Burroughs et al., '51) was employed. It was evident that additional information regarding the nutritional requirements of rumen microorganisms could be obtained only if an active inoculum washed as free as possible of rumen liquid was used.

Various compounds or groups of compounds have been reported to stimulate cellulose digestion by rumen microorganisms *in vitro*. These include glucose (Hofflund et al., '48) various water-soluble vitamins, purines and pyrimidines

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trifuged at high speed ( $25,000 \times G$ ) for 25 minutes to sediment bacterial cells. The cells were resuspended in a solution containing glucose, cysteine and the same salts as were present in the basal medium. The concentrations of the latter in the wash solution were the same as those in the basal medium while glucose was present at a concentration which would provide the optimum level of glucose to the fermentation medium when the inoculum was added to the tubes. The resuspended cells were centrifuged, the supernatant removed and the cells again suspended in the wash solution. This operation was repeated twice. The cells were finally diluted with a volume of wash solution usually equal to one-fifth of the volume of the rumen liquid from which the cells were originally obtained and 2.5 ml of this suspension was added to each assay tube.

To ensure that the inocula used from assay to assay had approximately the same initial activity, a means of comparing the activity of the various inocula was developed. The time required for a given volume of the washed suspension of rumen microorganisms to reduce a solution of triphenyl tetrazolium chloride under standard conditions was determined. In this test 2.5 ml of the washed suspension was added to 2.5 ml of a solution containing 0.25 ml of 0.1% triphenyl tetrazolium chloride, 0.3 ml of 10% glucose and 1.0 ml of M/15 phosphate buffer. The time required to reduce the dye at 40° C. was then determined. Reduction times obtained with the inocula were correlated with the rates of the corresponding fermentations. Inocula reducing the dye in times ranging from 4 to 6 minutes were found to be the most satisfactory. Suitable adjustments in the volume of the suspensions could usually be made to obtain inocula having reducing times falling within this range.

**Basal medium.** The composition of the chemically defined medium found in this study to be capable of promoting the best cellulose digestion by rumen microorganisms *in vitro* is presented in table 1. Cellulose was added to the medium as a roll of vegetable parchment. Glucose was incorporated

(Hall et al., '53; Bentley et al., '54), an amino acid mixture (MacLeod and Brumwell, '54), urea (Belasco, '54), some steroid compounds (Brooks et al., '54) and certain short-chain fatty acids (Bentley et al., '55; Bryant and Doetsch, '55).

Using a well-washed inoculum of rumen microorganisms, a chemically defined medium has been developed incorporating at their optimum levels those factors found to be effective in this study in stimulating cellulose digestion. The medium developed was then used to determine whether various natural materials have a capacity to produce a further stimulation of cellulose digestion. In the course of this study it was established that three amino acids, namely leucine, isoleucine and valine were primarily responsible for the strong stimulation of cellulose digestion previously shown to be produced by a mixture of 18 amino acids (MacLeod and Brumwell, '54).

#### EXPERIMENTAL METHODS

In vitro rumen fermentations were carried out in a series of  $18 \times 150$  mm test tubes essentially as described previously (MacLeod and Brumwell, '54). Cellulose digestion was measured by determining the difference in the weight of a roll of vegetable parchment (dialyzer paper) before and after fermentation.

*Preparation of the inoculum.* Inocula washed with varying degrees of thoroughness have been employed in certain of the more recent studies of cellulose digestion by rumen microorganisms *in vitro* (Bentley et al., '55; Cheng et al., '55).

The following procedure, developed in this laboratory, was found to give rise to a cellulolytically active, washed inoculum of rumen microorganisms. Rumen liquid obtained at an abattoir from the paunches of freshly killed cattle was strained through cheese-cloth to remove gross particles. The strained liquid was centrifuged at low speed ( $125 \times G$ ) in a Servall SS-1 centrifuge for 5 minutes to remove as much non-bacterial matter as possible. The supernatant liquid was then cen-

trifuged at high speed ( $25,000 \times G$ ) for 25 minutes to sediment bacterial cells. The cells were resuspended in a solution containing glucose, cysteine and the same salts as were present in the basal medium. The concentrations of the latter in the wash solution were the same as those in the basal medium while glucose was present at a concentration which would provide the optimum level of glucose to the fermentation medium when the inoculum was added to the tubes. The resuspended cells were centrifuged, the supernatant removed and the cells again suspended in the wash solution. This operation was repeated twice. The cells were finally diluted with a volume of wash solution usually equal to one-fifth of the volume of the rumen liquid from which the cells were originally obtained and 2.5 ml of this suspension was added to each assay tube.

To ensure that the inocula used from assay to assay had approximately the same initial activity, a means of comparing the activity of the various inocula was developed. The time required for a given volume of the washed suspension of rumen microorganisms to reduce a solution of triphenyl tetrazolium chloride under standard conditions was determined. In this test 2.5 ml of the washed suspension was added to 2.5 ml of a solution containing 0.25 ml of 0.1% triphenyl tetrazolium chloride, 0.3 ml of 10% glucose and 1.0 ml of M/15 phosphate buffer. The time required to reduce the dye at  $40^{\circ}C$ . was then determined. Reduction times obtained with the inocula were correlated with the rates of the corresponding fermentations. Inocula reducing the dye in times ranging from 4 to 6 minutes were found to be the most satisfactory. Suitable adjustments in the volume of the suspensions could usually be made to obtain inocula having reducing times falling within this range.

*Basal medium.* The composition of the chemically defined medium found in this study to be capable of promoting the best cellulose digestion by rumen microorganisms *in vitro* is presented in table 1. Cellulose was added to the medium as a roll of vegetable parchment. Glucose was incorporated



into the medium with the inoculum. The mixture of salts used was the same as that employed previously (MacLeod and Brumwell, '54). The remaining components of the medium were added at levels established by experiment to be optimum under the conditions employed in this study.

TABLE 1  
*Composition of fermentation medium*

COMPONENT	AMOUNT/20 ml
Cellulose	500 mg
Glucose	20 mg
Urea	18.45 mg
Cysteine	15 mg
Amino acid mixture <sup>1</sup>	4.0 ml
Vitamin mixture <sup>2</sup>	1.0 ml
Salts A <sup>3</sup>	1.71 ml
Salts B <sup>4</sup>	0.17 ml
CaCl <sub>2</sub>	0.646 mg

<sup>1</sup> Amino acid mixture: DL-alanine, 2 gm; L-aspartic acid, 400 mg; L-glutamic acid, 1 gm; DL-threonine, 400 mg; DL-serine, 400 mg; glycine, 200 mg; L-lysine, 400 mg; L-methionine, 200 mg; L-cystine, 200 mg; L-arginine, 400 mg; L-proline, 200 mg; L-histidine, 200 mg; DL-phenylalanine, 400 mg; L-tyrosine, 200 mg; L-tryptophan, 200 mg; L-valine, 200 mg; L-leucine, 200 mg; L-isoleucine, 400 mg. in total volume of 500 ml.

<sup>2</sup> Vitamin mixture: riboflavin, 10 mg; pyridoxal, 2 mg; calcium pantothenate, 10 mg; thiamine, 10 mg; niacin, 10 mg; *p*-aminobenzoic acid, 2 mg; biotin, 100 µg; folic acid, 100 µg; vitamin B<sub>12</sub>, 15 µg, in a total volume of 100 ml.

<sup>3</sup> Salts A: Na<sub>2</sub>HPO<sub>4</sub>, 86 gm; NaHCO<sub>3</sub>, 26.25 gm; KCl, 3.75 gm; NaCl, 3.75 gm; MgSO<sub>4</sub>, 1.30 gm; in a total volume of 1000 ml.

<sup>4</sup> Salts B: FeSO<sub>4</sub>·(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>·6H<sub>2</sub>O, 3.89 gm; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.80 gm; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.43 gm; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.625 gm; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.337 gm, in 1000 ml of solution.

*Fermentation technique.* The technique employed in this investigation was only a slight modification of that described previously (MacLeod and Brumwell, '54). In the present investigation smaller test tubes (18 × 150 mm) were used. Each tube in each assay was prepared in duplicate. Before inoculation CO<sub>2</sub> gas was bubbled through the contents of each tube to produce anaerobic conditions and at the same time to lower the pH of the medium to the starting value of 6.9. To maintain anaerobic conditions and still provide a

means of making allowances for the production of fermentation gases, the tubes were capped with small rubber balloons.

It was found in preliminary studies that there was no particular advantage to adjusting the pH of the tubes during the course of the fermentation so long as the fermentation did not proceed too far. In this study the fermentation was terminated when visual inspection indicated that approximately 30 to 50% of the cellulose had digested in those tubes which were fermenting most rapidly.

#### EXPERIMENTAL

*Response to amino acids.* The previous study had revealed that cellulose digestion by rumen microorganisms was strongly stimulated by the addition of a mixture of 18 amino acids to the fermentation medium (MacLeod and Brumwell, '54). A similar response to amino acids was obtained using a washed inoculum of rumen microorganisms and the level of the mixture producing maximum stimulation in the medium was approximately the same as found previously to be optimum. To determine which of the amino acids in the mixture were responsible for the effect obtained, the 18 amino acids were first subdivided into three groups of 6 each. These groups were tested alone and in combination for their ability to promote cellulose digestion. It can be seen, table 2, that only one of the three groups, group C, was active and in this experiment it proved to be more active than the complete mixture. When the 6 amino acids in group C were divided into the two sub-groups C<sub>1</sub> and C<sub>2</sub>, sub-group C<sub>2</sub> was found to have the same activity as that of C. Sub-group C<sub>2</sub> contained leucine, isoleucine and valine. Omission of each of the amino acids in turn from group C<sub>2</sub> indicated that each was making a contribution to the response produced by the mixture in this experiment.

Because the types of organisms composing the rumen population can be present in the rumen in differing proportions (Gall et al., '53) especially if the feeding regimen of the animals is not well controlled, one might expect some varia-

tion in the response of the population to growth factors from one time to another. For this reason, experiments of the type illustrated were repeated using inocula obtained from rumen liquid collected on numerous different occasions. On each occasion the sub-group containing leucine, isoleucine and valine was found to be responsible for most or all of the activity of the complete mixture. When the three amino acids

TABLE 2

*The effect of groups of amino acids on cellulose digestion by rumen microorganisms in vitro*

ADDITIONS TO MEDIUM <sup>1</sup>	% CELLULOSE DIGESTED <sup>2</sup>
None	7
Complete mixture	23
Group A	8
Group B	3
Group C	35
Group C <sub>1</sub>	6
Group C <sub>2</sub>	32
C <sub>2</sub> minus valine	18
C <sub>2</sub> minus leucine	8
C <sub>2</sub> minus isoleucine	12

<sup>1</sup> The medium of table 1 was used with the amino acid mixture omitted and the total nitrogen content of each tube maintained at 17.5 mg by the addition of an appropriate level of urea.

The various groups shown contained the following amino acids: A — alanine, aspartic acid, glutamic acid, threonine, serine, glycine; B — lysine, methionine, cystine, arginine, proline, histidine; C — phenylalanine, tyrosine, tryptophan, valine, leucine, isoleucine; C<sub>1</sub> — phenylalanine, tyrosine, tryptophan; C<sub>2</sub> — valine, leucine, isoleucine.

<sup>2</sup> Incubation time = 72 hours.

were tested alone and in combination on the different occasions, however, somewhat more variable results were obtained. Usually the three amino acids together were better than each tested singly or in pairs. In one case, however, the three amino acids were all equally active when tested individually while in another certain pairs were as good as all three. At no time was it found possible to obtain appreciable cellulose digestion without any of the three amino acids being present.

The results of the experiment recorded in table 2 indicate that in this case the complete amino acid mixture was less effective than the combination of leucine, isoleucine and valine in promoting cellulose digestion. In general, however, the complete mixture had at least the same and sometimes slightly more activity than the more limited number of amino acids. For this reason the complete mixture of amino acids rather than the combination of leucine, isoleucine and valine alone was included routinely in the preparation of the basal medium.

TABLE 3

*A comparison of the ability of certain amino acids and fatty acids tested alone and in combination to promote cellulose digestion by rumen microorganisms in vitro*

ADDITIONS TO MEDIUM <sup>1</sup>	% CELLULOSE DIGESTED <sup>2</sup>
None	20
Valine + leucine + isoleucine (12 $\mu$ M of each)	51
Valine + leucine + isoleucine (24 $\mu$ M of each)	47
Valeric + isovaleric acids (12 $\mu$ M of each)	38
Valine + leucine + isoleucine + valeric acid + isovaleric acid (12 $\mu$ M of each)	44

<sup>1</sup> See table 2.

<sup>2</sup> Incubation time = 91 hours.

Bentley et al. ('55) found that cellulose digestion by rumen microorganisms could be stimulated by certain short-chain fatty acids present in rumen liquid as well as by proline and valine. Bryant and Doetsch ('55) observed that *Bacteroides succinogenes*, a cellulolytic bacterium isolated from the bovine rumen, required a combination of a 5- to 8-carbon straight-chain fatty acid and a branched-chain fatty acid which could be either isobutyric, isovaleric or dl-2-methyl-n-butyric acid for growth. It was of interest to know whether the effect of these volatile fatty acids on cellulose digestion by rumen microorganisms was related to or independent of the response of the organisms to leucine, isoleucine and valine.

The results in table 3 show the response to a combination of valine, leucine and isoleucine when these were tested at the level at which they were present in the complete mixture used in the basal medium. Doubling the concentration of the amino acids had a slightly inhibitory effect. Valeric and isovaleric acids tested in combination at the same level as the three amino acids promoted cellulose digestion but not to quite the same extent as the amino acids. The addition of the fatty acids to a medium containing the three amino acids caused no further stimulation of cellulose digestion but actually a decrease similar to that obtained by adding an excess of the three amino acids.

There was thus no evidence of synergism when the amino acids and the short-chain fatty acids were tested together, but rather it would appear from these results that the two types of compounds were being used interchangeably in promoting cellulose digestion by the rumen microorganisms.

*Response to vitamins.* Hall et al. ('53) found that biotin and vitamin B<sub>12</sub> were stimulatory to cellulose digestion *in vitro*, while Bentley et al. ('54) reported a response to biotin, vitamin B<sub>12</sub> and *p*-aminobenzoic acid.

A mixture of 9 vitamins when added to the fermentation medium used in this study consistently stimulated cellulose digestion by the rumen microorganisms. Some difficulty, however, was encountered in establishing which vitamins singly or in combination contributed to the stimulation produced by the mixture. The results of one experiment, shown in table 4, reveal that on this occasion the omission of either pyridoxal, thiamine or niacin lowered the response of the micro-organisms to the vitamin mixture. In other experiments folic acid and *p*-aminobenzoic acid also appeared to play a role. Throughout these experiments, pyridoxal (replaceable by pyridoxamine or pyridoxine) was consistently effective in stimulating cellulose digestion. To ensure maximum stimulation from the water-soluble vitamins, the complete mixture was included in the basal medium at a level slightly in excess of that producing an optimum response.

*Response to glucose.* Hoflund et al. ('48) found that 0.1 to 0.2% of glucose in the medium stimulated cellulose digestion by rumen microorganisms *in vitro* while higher levels depressed it.

The effect of adding increasing concentrations of glucose to the fermentation medium used here is shown in table 5. In this experiment glucose was omitted from the wash solution used to prepare the inoculum.

TABLE 4

*Effect of vitamins on cellulose digestion by rumen micro-organisms*

ADDITIONS TO MEDIUM <sup>1</sup>	% CELLULOSE DIGESTED <sup>2</sup>
None	20
Complete vitamin mixture	36
minus riboflavin	33
minus pyridoxal	27
minus calcium pantothenate	35
minus thiamine	27
minus niacin	29
minus <i>p</i> -aminobenzoic	34
minus biotin	36
minus vitamin B <sub>12</sub>	34
minus folic acid	35

<sup>1</sup> The medium of table 1 was used with the vitamin mixture omitted.

<sup>2</sup> Incubation time = 64 hours.

TABLE 5

*Effect of increasing levels of glucose on cellulose digestion by rumen microorganisms in vitro*

GLUCOSE ADDED <sup>1</sup>	% CELLULOSE DIGESTED <sup>2</sup>
0	19
10	25
20	24
30	29
40	24
50	22
100	19

<sup>1</sup> The medium of table 1 was used with glucose omitted.

<sup>2</sup> Incubation time = 64 hours.

The results show that stimulation of cellulose digestion was obtained in the same range of glucose concentrations as that observed by Hoflund et al. The optimum response was obtained over a range of 10 to 40 mg per tube (0.05 to 0.2%) while decreased effects were observed at higher concentrations. Although the magnitude of the stimulation was not great, it was found that the routine inclusion of glucose at a 0.1% level in the medium greatly improved the reproducibility of all assay results.

TABLE 6

*The relation of the cellulolytic factor added to the response of rumen microorganisms to urea*

UREA ADDED <sup>1</sup>	CELLULOLYTIC FACTOR ADDED		
	Isovaleric + valeric acids	Amino acids Restricted <sup>2</sup>	Complete <sup>3</sup>
mg/20 ml	% cellulose digested <sup>4</sup>		
0	11	16	27
18	27	26	36
27	30	35	38
36	32	39	42
45	34	38	25
54	28	23	0

<sup>1</sup> The basal medium used was that of table 1 with urea and the amino acid mixture omitted.

<sup>2</sup> Leucine + isoleucine + valine added at the level present in the complete mixture.

<sup>3</sup> Complete mixture of 18 amino acids (see table 1).

<sup>4</sup> Incubation time = 89 hours.

*Response to urea.* The presence of urea along with various food proteins in *in vitro* rumen fermentation studies has been found to improve cellulose digestion over that observed with the food proteins alone (Belasco, '54). In the present study, the level of urea needed for maximum stimulation of cellulose digestion varied depending on the "cellulolytic factor" employed. More was required if the combination of valeric and isovaleric acids was added than if amino acids were used, table 6. Even in the presence of the mixture of 18 amino acids the addition of urea was quite strongly stimulatory. Further increases in urea concentration beyond the level re-

quired for maximum activity brought about a decrease in the amount of cellulose utilized, in a manner similar to that observed by Belasco ('54). In the presence of the complete amino acid mixture, not only was less urea required for maximum cellulose digestion but also less was needed to produce inhibition. Thus there is a comparatively narrow range of nitrogen concentration producing optimum cellulose digestion, a factor to be considered when testing the growth-promoting activity of natural materials.

TABLE 7

*Effect of various supplements on cellulose digestion by rumen microorganisms in the complete synthetic medium*

SUPPLEMENT TO SYNTHETIC MEDIUM <sup>1</sup>	EXP. 1	EXP. 2	EXP. 3
	% cellulose digested		
None	33	41	46
Whale solubles	42	45	52
Herring solubles	35	41	54
Yeast extract	37	40	44
Enzymatic casein	36	41	36
Beef liver extract	41	48	
Malt extract	28	38	
Herring stickwater			51

<sup>1</sup> The complete medium of table 1 was used as the basal medium. Each supplement was tested at a series of levels providing from 1.7 to 12.1 mg of nitrogen per tube.

*Effect of miscellaneous supplements.* Purines and pyrimidines as well as various steroid compounds have been reported to stimulate digestion by rumen microorganisms *in vitro* (Bentley et al., '54; Brooks et al., '54). Adenine, guanine, uracil and xanthine added alone and in combination to this basal medium at a level of 400 µg per tube had no stimulatory effect on the fermentation. The addition of cholesterol at a level of 400 µg per 20 ml of medium both alone and in combination with Tween 40 likewise did not affect cellulose digestion under the conditions used here.

*Addition of natural materials to the fermentation medium.* When all of the compounds found in this study to be active in



promoting cellulose digestion were combined at levels producing maximum activity, the medium shown in table 1 was obtained. To determine whether additional factors were active in stimulating cellulose digestion, supplements of various natural materials were added to the synthetic medium. The results obtained in three different experiments are summarized in table 7. Since the margin between the optimum and the inhibitory level of nitrogen in the medium was small and somewhat variable, each supplement was tested at a number of different levels in each experiment. The level of supplement at which a maximum response was obtained varied from one experiment to the next. The results reported in table 7 represent the maximum response obtained for each supplement in each experiment. It can be seen that certain of the supplements, particularly whale solubles and beef liver extract produced an appreciable stimulation of cellulose digestion over that obtained in the synthetic medium alone. The amounts of supplement required to produce a response, however, were quite large, ranging from 30 to 60 mg per tube.

#### DISCUSSION

In this study leucine, isoleucine and valine have been found to be interchangeable with short-chain fatty acids in promoting cellulose digestion by rumen microorganisms *in vitro*. Bentley et al. ('55) have found valine to be active in stimulating cellulose digestion in the absence of fatty acids. In studying the growth requirements of *Bacteroides succinogenes*, a cellulose digesting microorganism isolated from the bovine rumen, Bryant and Doetsch ('55) obtained a response of the organism to short-chain fatty acids in a medium containing an enzymatic digest of casein. The same brand of enzymatic casein at the level used by the latter authors has been found in this study to produce maximum cellulose digestion by rumen microorganisms and all attempts to increase the extent of digestion by adding either leucine, isoleucine and valine or short chain fatty acids have been unsuccessful. The difference in response obtained can probably be ascribed

to the fact that in the cases where amino acids and fatty acids have been found to be interchangeable a mixed culture of rumen microorganisms was used. Hungate ('50) has described 7 and Huhtanen and Gall ('53) 9 different bacteria or strains of bacteria isolated from the bovine rumen capable of digesting cellulose or fibre. It is of course possible that fatty acids are able to stimulate one and the amino acids another of these strains or group of strains. Knowledge of the growth requirements of other cellulolytic bacteria which have been isolated from the rumen in pure culture should prove of interest in connection with this possibility. The similarity in structure of the active amino acids and fatty acids, however, appears to be more than coincidental and it is likely that, in the mixed culture, organisms associated with the cellulose digesters can convert the appropriate amino acids to the specific fatty acids required by at least one strain of cellulose digesting microorganism. Evidence for the production of volatile fatty acids from amino acids by microorganisms from the rumen of sheep has been demonstrated by El-Shazly ('52). Of particular interest in connection with the work reported here is the fact that the latter author has postulated that the branched-chain  $C_4$  and  $C_5$  volatile fatty acids in the rumen are formed from valine, leucine and isoleucine as a result of Stickland type reactions.

Whether the response to supplements of natural materials, which was obtained in the fermentation, is due to one or more specific factors present in the supplements or to a more generalized response to the addition of large quantities of preformed growth factors to the medium has not been established. The relatively large amounts of the supplements required to obtain the responses observed suggests that the latter explanation could account for the effect. To make it profitable to pursue this phase of the problem further inocula obtained under more reproducible conditions than are presently available to this laboratory would be required.

## SUMMARY

Using a washed inoculum of rumen microorganisms a chemically defined medium has been developed incorporating at their optimum levels those factors found to be effective in this study in stimulating cellulose digestion. In this medium responses to amino acids, short-chain fatty acids, various vitamins, glucose and urea have been demonstrated.

A combination of valine, leucine and isoleucine was found to be primarily responsible for the strong stimulation of cellulose digestion previously shown to be produced by a mixture of 18 amino acids. The combination of leucine, isoleucine and valine could be used interchangeably with and was somewhat more effective than the volatile fatty acids tested in promoting cellulose breakdown.

Of the various vitamins tested, vitamin B<sub>6</sub> was the most consistent in its ability to stimulate cellulose digestion by rumen microorganisms in the medium used.

The level of urea required for maximum stimulation of cellulose digestion varied depending on the cellulolytic factor employed. More was required if the combination of valeric and isovaleric acids was added than if amino acids were used. Optimum cellulose digestion occurred over a comparatively narrow range of nitrogen concentration in the medium. Above this range inhibition occurred.

When various natural materials, in particular whale solubles and beef liver extract, were added to the synthetic medium, some further stimulation of cellulose digestion over that obtained in the synthetic medium alone resulted. Relatively large amounts of the supplements were required to produce the additional response.

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# THE AMINO ACID REQUIREMENT OF THE LAYING HEN

## I. THE DEVELOPMENT OF A FREE AMINO ACID DIET FOR MAINTENANCE OF EGG PRODUCTION<sup>1</sup>

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Very little is known about the amino acid requirements of the laying hen (Bird et al., '54; Almquist, '52). This paucity of information has been primarily the result of an inability to formulate a free amino acid diet on which hens would maintain egg production. Attempts to formulate such a diet have been reported only by Wisconsin workers (Ingram et al., '50a) who found that egg production ceased in two to 4 days when hens were fed amino acid mixtures. Grau and associates ('48, '49) have also reported difficulties with purified diets for laying hens.

It is the purpose of the present report to discuss studies from this laboratory that have led to the successful formulation of a free amino acid diet suitable for studying the qualitative and quantitative amino acid requirements of the chicken for egg production.

### EXPERIMENTAL

Single Comb White Leghorn hens from the University flock which were laying in clutches of two eggs or more were used

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in these studies. The birds were maintained in individual cages in a temperature regulated room and allowed water ad libitum. When not on an experimental diet, the hens were fed an all-mash ration.<sup>2</sup>

In view of the findings by Almquist ('47) that chicks fed free amino acid diets exhibited depressed appetites, birds were force-fed in the early experiments in order to maintain a constant nutritive intake for body maintenance and egg formation. Force-feeding was carried out at the rate of

$$Y = 37 W^{.7}$$

where

$$Y = \text{grams of diet per day}$$

and  $W$  = body weight to the nearest 0.1 lb. at the beginning of an experiment. This formula is based on an arbitrary feed intake of 25 gm per pound body weight for a 3.6 lb. bird, laying at a normal rate.

The force-feeding was accomplished with the aid of a metal funnel attached to a  $\frac{3}{8}$  in. polyethylene tube, the latter extending into the crop. The diet was mixed in a beaker with equal parts of water by weight and was then poured into the funnel. Diet adhering to beaker and funnel was rinsed out with a wash bottle. When force-feeding was employed, birds were allowed access to the diet and were fed 6 times per day at two-hour intervals.

Five different basal diets were employed in the development of the adequate diet and are given in table 1. The amino acids essential for the chick (Almquist and Grau, '44), arginine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine together with alanine, aspartic acid, cystine, proline, serine and tyrosine were used to develop the diet. The amino acid components were thoroughly mixed with the basal diet preceding each experimental trial and the total made up to one

<sup>2</sup> Ground yellow corn, 35.5; ground wheat, 20; ground oats, 20; soybean oil meal, 5; meat scrap, 7.5; alfalfa meal, 6; steamed bone meal, 2; mineral concentrate (Mico. Limestone Corporation of America), 3; salt, 0.35; vitamin A & D oil, 0.4; vitamin B<sub>12</sub>-antibiotic feed supplement, 0.25.

hundred parts with the addition of glucose (cerelose) or starch. Sodium bicarbonate was added to neutralize the HCl radicals of the basic amino acids on a molecular basis at the beginning of the study; later bicarbonate addition was standardized at 1% in all diets.

Two birds were used per treatment; these were repeated until satisfactory conclusions could be drawn.

TABLE 1  
*Composition of basal diets*

INGREDIENT	DIET				
	A	B	C	D	E
	%	%	%	%	%
Glucose (cerelose)	56.96	31.96	18.96	.....	.....
Corn starch		20.00	30.00	51.96	51.96
Corn oil	5.00	5.00	8.00	10.00	12.00
Fiber	5.00	5.00	5.00	5.00	3.00
Mineral mix <sup>1</sup>	5.34	5.34	5.34	5.34	5.34
Lime-stone	2.50	2.50	2.50	2.50	2.50
Vitamin A, D and E concentrate <sup>2</sup>	0.10	0.10	0.10	0.10	0.10
Choline Cl	0.10	0.10	0.10	0.10	0.10
Vitamin mix <sup>3</sup>	0.15	0.15	0.15	0.15	0.15
Variable ingredients (amino acids, antacid adsorbent, <sup>4</sup> bicarbonate, starch)	24.85	20.85	20.85	24.85	24.85
	100.00	100.00	100.00	100.00	100.00

<sup>1</sup>Percentage of diet:  $\text{CaCO}_3$ , 0.3000;  $\text{Ca}_3(\text{PO}_4)_2$ , 2.8000;  $\text{K}_2\text{HPO}_4$ , 0.0070;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2500;  $\text{Fe}(\text{C}_2\text{H}_3\text{O}_2)_3 \cdot 6\text{H}_2\text{O}$ , 0.1400;  $\text{ZnCl}_2$ , 0.0020;  $\text{KI}$ , 0.0040;  $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.0020;  $\text{H}_3\text{BO}_3$ , 0.0002;  $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0001;  $\text{MnSO}_4$ , 0.0050;  $\text{NaCl}$ , 0.8800.

<sup>2</sup>Supplies per kilogram of diet: 10,000 IU vitamin A, 600 IU vitamin D, and 5 IU alpha-tocopheryl acetate.

<sup>3</sup>In milligrams per kilogram of diet: thiamine HCl, 25; riboflavin, 16; Ca pantothenate, 20; vitamin B<sub>6</sub>, 0.62; pyridoxine HCl, 5; niacin, 0.6; folic acid, 4; inositol, 100; pantothenic acid, 2; 2-methyl-5-pyridyl pyrazole, 5; ascorbic acid, 100; choline, 250.

<sup>4</sup>Adsorbent: aluminum hydroxide-magnesium hydroxide preparation, a gift of Warrick Chemicals Corp., New York, N. Y.



## RESULTS

The first amino acid mixture to be tried with basal diet A was based on the published requirements for the growing chick. The force-feeding of this diet resulted in immediate stasis of the digestive tract and coma developed after two days. Upon being sacrificed, the birds showed hemorrhagic and necrotic areas in the liver. Crop and proventriculus were also affected, as indicated by many bleeding ulcers. The stasis of the digestive tract was similar to that described by Spector and Adamstone ('50) in rats on an amino-acid-deficient diet.

Noticeable improvement in the general condition of the birds was achieved upon the addition of higher levels of isoleucine and lysine as well as by a change in the source of the amino acids.<sup>3</sup> Supplementation with 1% of an aluminum hydroxide-magnesium trisilicate preparation,<sup>4</sup> originally introduced to control the ulcer condition, further improved the diet. With these changes, coma, liver necrosis and ulcer formation were prevented and food passage was normal; nevertheless, egg production always ceased within 4 days, as had been the experience of Ingram et al. ('50a) under ad libitum feeding conditions.

*Caloric intake.* The greatest improvements toward a successful ration were made by increasing the energy content. Basal diet B contained 20% starch added at the expense of an equal amount of glucose (cerelose).<sup>5</sup> This change resulted in continued egg production for 6 to 7 days instead of the 4 day limit previously observed. In basal diet C, energy was further increased by raising the starch level at the expense of glucose, as well as by a substantial increase in the corn oil from 5 to 8%. On this diet one bird maintained egg production for the entire two-week experimental period, laying 10

<sup>3</sup> With the exception of glycine, arginine, and methionine, all amino acids were henceforth purchased from Nutritional Biochemicals Incorporated, Cleveland, Ohio.

<sup>4</sup> Gelusil, courtesy of Warner-Chileott Laboratories, New York, N. Y.

<sup>5</sup> The change provided a small increase in caloric intake since Anderson and Hill ('55) have indicated that cornstarch provides 10% more metabolizable energy than glucose (cerelose).

eggs in 14 days. To the authors' knowledge, this is the first report of a hen maintaining normal production for this length of time on a diet of free amino acids.

As a result of these experiments it was apparent that most of the difficulty in maintaining egg production on amino acid diets was related to the caloric intake of the birds. Rose, Coon and Lambert ('54) have shown that humans require progressively more energy to maintain positive nitrogen balance as the diet is changed from casein to hydrolyzed casein to an amino acid mixture.

Basal diet D was next employed; it contained starch as the only carbohydrate and 10% of corn oil. It soon became evident that this diet was rather well balanced, since most, but not all, hens began eating it by themselves at a normal rate, at the same time maintaining normal egg production over the two-week experimental period.<sup>6</sup>

A typical experiment showing *ad libitum* feed consumption and egg production for 10 hens on this diet is shown in table 2. It will be noticed that all birds that ate well *throughout* the experimental period continued to lay, while those that did not consume enough nutrients did not maintain production. This was confirmed in several other experiments not listed here. Thus, the adequacy of a diet seems to be reflected in the voluntary feed consumption of hens during the first week. Egg production may not be affected until the second week due to the presence of ova in various stages of development as well as other protein stores. This is best illustrated in table 3 which demonstrates that birds on imbalanced diets laid only a few eggs and nearly all of them stopped production within the first week. In view of these consistent findings, a two-week experimental period was chosen for these studies.

To determine the optimum dietary energy level, 12 and 15% corn oil additions were next studied. The 15% level

<sup>6</sup> The quality studies revealed no differences in size or quality before, during, or after the experimental period. Rogers et al. (1955) have found that dietary changes in amino acids do not affect egg quality.

of corn oil appeared too high, since birds placed on this diet consumed less feed and laid at a much reduced rate. On the other hand, the birds on the 12% corn oil level laid at a good rate (9 eggs/bird/14 days), their production being signifi-

TABLE 2

*Egg production and feed consumption on a diet of free amino acids*

BIRD NO.	DAILY EGG PRODUCTION DURING 14-DAY EXPERIMENTAL PERIOD														TOTAL EGGS
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
861	x	x	x											x	4
899	x	x	x		x	x	x		x	x		s <sup>1</sup>			9
887	x	x		x	x	x				x					6
883	x		x	x	x		x	x		x	x	x	x		10
897		x	x		x		x	s		x	s		x	x	9
873	x	x	x		x	x			x	x	x	x	x	x	11
865	x		x	x		x	x		x	x		x	x		9
868	x	x	x		x	x									5
880	x		x	x		x	x		x		x	x	x		9
898	x		x	x	x	x		x	x		x	x		x	10
DAILY FEED CONSUMPTION (gm/day)															AV.
861	6	25	30	77	83	106	93	86	84	90	86	87	83	85	73
899	102	87	72	75	98	98	91	101	99	77	81	78	93	86	88
887	68	59	62	92	84	61	102	76	70	66	75	83	105	81	77
883	54	75	86	86	95	107	113	93	100	100	95	78	100	95	91
897	56	67	34	97	109	98	100	101	97	109	100	97	129	102	93
873	48	78	105	120	101	79	142	109	118	120	113	89	88	103	101
865	65	41	73	68	100	92	77	88	62	59	74	77	63	67	72
868	31	41	55	63	31	52	57	21	27	50	60	82	61	51	49
880	0	48	72	51	117	68	136	100	51	108	96	63	122	95	80
898	55	110	108	108	107	75	108	116	94	98	98	74	88	91	95

<sup>1</sup> Soft shell egg.

TABLE 3

*The effect of dietary amino acid and energy imbalances on the number of eggs laid*

NUMBER OF EGGS LAID BEFORE PRODUCTION CEASED	NUMBER OF BIRDS	DAYS BEFORE PRODUCTION CEASED
1	4	1-2 <sup>1</sup>
2	12	2-4
3	20	4-6
4	8	5-7
5	1	8
6	1	9

<sup>1</sup> Range.

cantly greater ( $P < 0.05$ ) than that of the birds on the 15% corn oil diet. Basal diet E containing 12% corn oil was therefore adopted as the basis of a ration which would contain a balance of amino acids suitable for the maintenance of egg production in a large proportion of the birds.

TABLE 4  
*Composition of successful free amino acid diet*

INGREDIENT		AMINO ACIDS <sup>2</sup>	
	%		%
Corn starch	55.91	DL-alpha Alanine	1.0
Corn oil	12.00	L-Arginine HCl	1.3
Fiber	3.00	L-Aspartic acid	0.5
Mineral mix <sup>1</sup>	5.34	L-Cystine	0.3
Limestone	2.50	L-Glutamic acid	3.5
Vitamin A, D and E concentrate <sup>1</sup>	0.10	Glycine	1.0
Choline Cl	0.10	L-Histidine HCl	0.6
Vitamin mix <sup>1</sup>	0.15	DL-Isoleucine	2.0
Antiacid adsorbent <sup>1</sup>	1.00	L-Leucine	1.4
Sodium bicarbonate	1.00	L-Lysine HCl (95%)	1.2
		DL-Methionine	0.4
		DL-Phenylalanine	1.0
	81.10	L-Proline	0.5
		DL-Serine	1.0
		DL-Threonine	1.0
		DL-Tryptophan	0.4
		L-Tyrosine	0.6
		DL-Valine	1.2
			18.9

<sup>1</sup> For composition see table 1.

<sup>2</sup> Gelusil, aluminum hydroxide-magnesium trisilicate.

<sup>3</sup> The arginine and glycine used were generously supplied by Merck & Co., Rahway, N. J.; DL-methionine was obtained through the courtesy of Dow Chemical Corp., Midland, Mich. All other amino acids were purchased from Nutritional Biochemicals Inc., Cleveland, Ohio.

Such a complete diet was developed and is listed in table 4. Typical results with this diet are shown in table 5. It can be seen that bird 878, which discontinued laying for 8 days, apparently consumed too little feed at the outset of the experiment; upon adjusting to a normal feed intake egg production was resumed. Thus, this diet is considered adequate

for maintaining normal egg production in most hens, enabling one to study the qualitative and quantitative amino acid requirements of the laying hen.

TABLE 5

*Egg production and feed consumption on a diet of free amino acids*

BIRD NO.	REPLI- CATE	DAILY EGG PRODUCTION DURING 14-DAY EXPERIMENTAL PERIOD														TOTAL EGGS	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		
857	1	x	x	x	x		x	x	x		x	x	x	x		11	
874		x			x		x		x	x		x	x		x	8	
908	2	x		x	x			x	x		x	x			x	8	
878			x	x									x		x	4	
DAILY FEED CONSUMPTION (gm/day)																	AV.
857	1	96	108	100	118	114	109	102	86	109	101	106	99	82	85	101	
874		49	87	94	79	84	93	89	71	84	80	71	61	61	65	76	
908	2	74	76	87	96	117	131	82	113	96	120	99	73	83	110	97	
878		23	21	27	78	101	94	118	107	123	122	134	118	126	126	94	

## DISCUSSION

The levels of amino acids used in this diet are not intended to represent the amino acid requirement of the laying hen; nor has an attempt been made to establish the most efficient levels of amino acids for egg production. Such data will be reported at a later time. Rather, the levels used in this diet represent only an acceptable balance of amino acids which will maintain egg production.

The level of lysine in particular is higher than the requirement of 0.52% of the diet reported by Ingram et al. ('51). Attempts to decrease the level below 0.91% (actual L-lysine) failed to maintain egg production. Calculations from practical diets would suggest that the requirement for L-lysine is not as great as the level used in this diet would indicate. This discrepancy requires further study to clarify the factors involved in the utilization of this amino acid.

Similarly, the isoleucine level of this diet is higher than the requirement for isoleucine as determined by Miller et al. ('54) on a practical diet.

Microbiological assay of the lysine and isoleucine samples yielded correct analytical values which suggests that impurities are not involved in this problem.

With the development of a complete diet that would maintain egg production, an attempt was made to evaluate the results obtained in the early studies. On repetition of these studies, with amino acids from a different source, it was found that the coma, necrotic liver, and also the ulcerative conditions were probably due to contamination in one or more amino acids. The possibility of heavy metal contamination and poisoning is suggested particularly by the liver condition.

Despite the fact that amino acids from a particular source seemed to account also for the ulcerative condition, it was interesting to note that an antacid adsorbent<sup>7</sup> continued to be a necessary constituent of the present diet. Attempts to eliminate it from the diet resulted in early stoppage of feed consumption and egg production. The beneficial effect of this product lies possibly in its magnesium content since Wisconsin workers (Heinicke et al., '56; Benton et al., '55) have shown the importance of added magnesium in the utilization of amino acids for the growth of the guinea pig and a similar growth-promoting effect in chicks on free amino acid diets upon the addition of the ash from gelatin.

Sodium bicarbonate was also necessary for the success of the diet. The action of sodium bicarbonate may be to neutralize the HCl radical on the basic amino acids, although Rose et al. ('50) have found in their human studies that neutralization of the acid group was not essential. Another action might be to bring the sodium-potassium ratio into more acceptable balance. Heinicke et al. ('56) have also demonstrated a beneficial effect from extra potassium supplementation on amino acid utilization in the guinea pig.

Because of the high cost of this diet, only a relatively small number of animals could be employed in each experiment. This has not been a serious handicap in this and the following studies since agreement among birds on the same treatment has generally been excellent. The two-week experimental period was chosen as sufficient time to demonstrate the adequacy of a diet to maintain egg production. As already demonstrated by Ingram et al. ('50a), hens receiving an incomplete diet will stop production in a very short period. That this finding was not restricted to free amino acid diets is illustrated in table 6 showing the same poor performance of

TABLE 6  
*Effect of amino acid-deficient protein diet on egg production*

BIRD NO.	TYPE OF DIET	EGG PRODUCTION	
		No added leucine	0.5% added leucine
882	15% Drackett protein	2 <sup>1</sup>	5 <sup>2</sup>
885	15% Drackett protein	3 <sup>1</sup>	8 <sup>2</sup>

<sup>1</sup> Stopped laying within 4-5 days.

<sup>2</sup> Number of eggs laid in 10-day period after recovery from leucine-deficient period; when returned to practical diet, production continued.

hens on a leucine-deficient soybean<sup>8</sup> protein diet. Later studies (Johnson and Fisher, '56) showed that birds could be continued on these amino acid diets for 30 days without any change in rate of production.

In accordance with existing custom, the amino acid content of the diet has been given as a percentage of the total diet. Although this expression of the amino acid levels is adequate here, especially since the weight range of the birds was a very narrow one, it is entirely unsatisfactory for quantitative expression. Energy requirement is proportional to a power of body weight; yet for maintenance purposes Rose and associates have never found any relationship between body weight

<sup>8</sup> Drackett Assay Protein. The Drackett Products Company, Cincinnati, Ohio.

or surface area and amino acid requirement. More important with regard to the hen, the amino acid requirements for a single egg are not dependent upon the body weight of the hen laying that egg but total feed consumption is dependent on body weight, which again emphasizes the importance of a better expression for the amino acid requirement than percentage of the diet. Therefore, it is proposed that the amino acid requirement for egg production be expressed in grams per day. The body weight and weight of eggs produced in a given length of time should be reported also to evaluate the effect of these factors on the total requirement. When enough data of this type become available in the future, the proper functions relating maintenance and production requirements can be formulated for each amino acid. This practice will be adopted in the quantitative aspects of this study in this laboratory.

Mention should be made of the "appetite" or "taste" for the amino acid diets exhibited by the hens. It seems abundantly clear that taste plays no important role in the consumption of these diets; nor does appetite appear to be anything but a reflection of the adequacy of the dietary balance. In this respect one is reminded of a similar response by many species of animals to certain B-vitamin deficiencies.

Egg production provides a very sensitive measurement of the amino acid requirements of the laying hen. The deposition of large quantities of protein as well as of other nutrients requires a delicate balance of many dietary factors. By means of the present diet these factors and their interrelationships may be studied critically using egg production as the sensitive criterion.

Besides the obvious application to the study of amino acid requirement, the diet lends itself equally well to a systematic study of the hen's requirement for other nutrients such as minerals, vitamins, and unidentified growth factors — a subject which is receiving much current attention by poultry nutritionists. In respect to unidentified factors required by the hen, several hens have been artificially inseminated after



they had been on an amino acid diet for 4 weeks. Eighty-three per cent fertility was obtained on a total of 12 eggs and of the 10 fertilized eggs, 9 normal chicks were hatched. Since it would seem unlikely that any unknown factors could be present in the free amino acid diets, the hen must either not require such factors for egg production and hatchability or else the bird has a sufficient store for at least a month.

#### SUMMARY

A free amino acid diet has been developed which was readily consumed in adequate amount by most hens with concomitant maintenance of normal egg production. The factors found to be of greatest importance in the formulation of this diet were the purity of amino acid source and the caloric level of the diet. In the latter respect, starch and 12% corn oil were used to maintain a high energy level with the amino acid mixture. Egg production provided an extremely sensitive criterion of amino acid requirement since on deficient diets production always ceased within one week. The usefulness of the free amino acid diet in studying amino acid requirements as well as other nutrients is emphasized.

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# THE AMINO ACID REQUIREMENT OF THE LAYING HEN

## II. CLASSIFICATION OF THE ESSENTIAL AMINO ACIDS REQUIRED FOR EGG PRODUCTION <sup>1</sup>

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Although the essential amino acid requirement for growth and maintenance in several animal species including man (Rose, '38; Almquist, '52; Rose et al., '55) has been the subject of considerable study, little information is available on the amino acid requirement for productive purposes as exemplified by milk production in the dairy cow and egg production in the hen. By injecting carbon 14-labeled bicarbonate and acetate intravenously into dairy cows, Black et al. ('52) indirectly classified those amino acids which could not be synthesized by the lactating dairy cow.

Lysine, methionine, tryptophan, leucine and isoleucine (Almquist, '52; Miller et al., '54) have been shown to be essential for egg production with the use of proteins deficient in one or more amino acids. The development of a free amino acid diet (Fisher and Johnson, '56) on which normal egg production could be maintained has made possible direct classification of all essential amino acids required for egg production.

### EXPERIMENTAL AND RESULTS

Single Comb White Leghorn hens from the university flock laying in clutches of two eggs or more were used in this study.

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Details of procedure and source of amino acids employed have been described previously (Fisher and Johnson, '56).

In the first experiment alanine, aspartic acid, cystine, glycine, proline and serine were simultaneously omitted from a mixture of 18 amino acids (Fisher and Johnson, '56). The nitrogen content of this diet in the first experiment and of all subsequent diets employed in the present study was standardized at 2% with the addition of ammonium citrate. The complete diet is shown in table 1. In table 2 detailed egg

TABLE 1

*Composition of complete diet for maintenance of egg production*

AMINO ACIDS <sup>1</sup>		BASAL DIET	
	%		%
L-Arginine HCl	1.3	Starch	58.41
L-Glutamic acid	3.5	Corn oil	12.00
L-Histidine HCl	0.6	Mineral mix <sup>2</sup>	5.34
DL-Isoleucine	2.0	Fiber	3.00
L-Leucine	1.4	Limestone	2.50
L-Lysine HCl (95%)	1.2	Dibasic ammonium citrate	1.50
DL-Methionine	0.8	Antacid adsorbent <sup>2</sup>	1.00
DL-Phenylalanine	1.0	Sodium bicarbonate	1.00
DL-Threonine	1.0	Vitamin A, D, and E concentrate <sup>2</sup>	0.10
DL-Tryptophan	0.4	Vitamin mix <sup>2</sup>	0.15
L-Tyrosine	0.6		
DL-Valine	1.2		

<sup>1</sup> Sources listed by Fisher and Johnson ('56).

<sup>2</sup> Composition given by Fisher and Johnson ('56).

production beyond the second week together with total production for a 30-day experimental period are given for three hens maintained on the modified diet and for one hen maintained on the 18 amino acid diet. Although a two-week experimental period had been shown previously to be fully satisfactory in determining the adequacy of free amino acid diets, it was felt necessary to demonstrate that birds could maintain egg production well beyond the two-week period.

The results in table 2 clearly demonstrate (a) that alanine, aspartic acid, citrulline, cystine, glycine, hydroxyproline, proline and serine are not essential for egg production when the

nitrogen intake is held at 2%; and (b) that hens will continue in production during a 30-day-experimental period.

Attempts to maintain egg production by replacing tyrosine with higher levels of DL-phenylalanine (2.0, 2.5, and 3.0%) were unsuccessful. At levels of 1.6 and 2.0% of the L-isomer of phenylalanine in place of the racemic form (DL), one out of two birds at each level maintained good egg production (7 and 8 eggs/bird/14 days, respectively) throughout the two-week experimental period. Despite the fact that tyrosine was only replaceable by phenylalanine with difficulty, it must be considered a non-essential amino acid (see discussion).

TABLE 2  
*Maintenance of egg production on free amino acid diets  
for a 30-day experimental period*

BIRD NO.	DAYS ON EXPERIMENT BEYOND 2 WEEKS																TOTAL EGGS FOR 30 DAYS
	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
12 amino acids in diet																	
900					x	x		x	x		x		x	x			14
966	x		x	x		x	x		x		x	x		x		x	20
905				x			x							x		x	10
18 amino acids in diet																	
852	x		x	x		x		x		x		x		x			15

The omission of glutamic acid from the amino acid mixture listed in table 1 did not interfere with ad libitum consumption of an adequate amount of feed (table 3). To date, however, no bird on a glutamic acid-free diet has maintained satisfactory egg production throughout the two-week experimental period; some hens stopped laying at the end of the first week and others maintained only a reduced rate of production (table 3). Glutamic acid must therefore be classified as an *essential* amino acid for egg production (in the presence of the amino acids listed in table 1) according to Rose's (38) definition of an essential amino acid.

Removal of any one of the remaining 10 amino acids (listed below) from the diet (table 1) resulted in immediate disrup-

tion of feed consumption. This observation demonstrates clearly the essentiality of each one of these amino acids. To obtain additional information regarding the effect on egg production of omitting these amino acids from the diet, the following experiment was designed: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were individually omitted from the otherwise complete diet (table 1). These diets were then force-fed to pairs of hens in adequate amounts during a 5-day period after which time the birds were returned to the prac-

TABLE 3

*Egg production and feed consumption on a glutamic acid-free diet*

BIRD NO.	DAILY EGG PRODUCTION DURING 14-DAY EXPERIMENTAL PERIOD														TOTAL EGGS
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
967		x	x		x			x				x		x	6
863	x	x	x				x		x				x		6
910	x		x		x	s <sup>1</sup>									4
874	x		x	x		x									4
DAILY FEED CONSUMPTION (gm/day)															AV.
967	54	54	68	94	94	115	119	120	116	111	113	71	82	85	93
863	70	60	78	104	95	81	56	60	56	35	24	53	42	57	62
910	88	96	106	122	120	116	77	103	88	117	82	95	98	100	101
847	10	24	28	91	93	135	102	113	97	132	91	78	71	92	83

<sup>1</sup> Soft shell egg.

tical laying ration. The time interval between the last egg on the experimental diet and the first egg on the practical diet offered critical proof of dietary essentiality. The data from this experiment are shown in table 4. The average pause in egg production due to the omission of *any* one of the amino acids listed was  $9.8 \pm 1.40$  days, while control hens continued laying throughout, with an average time interval (as explained above) of only  $1.9 \pm 1.50$  days. The difference between these means is highly significant with  $P < < 0.001$ . Therefore, the essential amino acids for egg production in the hen as determined in these studies are: arginine, glutamic acid, histi-

dine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

TABLE 4

*Effect on egg production of force-feeding for 5 days a diet from which an essential amino acid has been omitted*

AMINO ACID OMITTED	REPLICATE 1		REPLICATE 2	
	Number of eggs <sup>a</sup>	Pause in production	Number of eggs	Pause in production
		<i>days</i>		<i>days</i>
Arginine	2 <sup>b</sup>	9 <sup>c</sup>	2 <sup>b</sup>	12 <sup>c</sup>
Histidine	1	11	2	10
Isoleucine	2	10	3	10
Leucine	2	9	3	10
Lysine	2	8	3	died <sup>d</sup>
Methionine	3	9	2	9
Phenylalanine + tyrosine	4	8	2	12
Threonine	2	died <sup>e</sup>	2	9
Tryptophan	3	10	1	12
Valine	2	11	3	7
SUMMARY				
Average egg production, per hen	Complete diet <sup>f</sup> 2.6 ± 0.49		One amino acid omitted <sup>g</sup> 2.3 ± 0.71	
Average pause in production, days	1.9 ± 1.50		9.8 ± 1.40	

<sup>a</sup> Eggs laid during 5-day experimental feeding period.

<sup>b</sup> Days from last egg on experimental diet to first egg after return to practical diet.

<sup>c</sup> Died on last day of experimental period; liver and reproductive tract abnormal.

<sup>d</sup> Average of 10 birds.

<sup>e</sup> Average of birds listed above.

#### DISCUSSION

A comparison of the essential amino acids required for egg production with those required for growth and maintenance makes it evident that they do not differ markedly from those of the growing chick. Only glycine, which is essential for maximum growth, is not essential for egg production. Particularly interesting is the observation that glutamic acid is essential for maximum egg production, just as it has been shown necessary for maximum growth in chicks, mice and rats.



(Almquist and Grau, '44; Maddy and Elvehjem, '49; Rose et al., '48). Since glutamic acid is not required for maximum growth when the other non-essential amino acids are added to the 10 essential ones for the rat, Rose et al. ('48) classified glutamic acid as a dispensable amino acid, although it is considered an *essential* component of diets containing only the 10 essential amino acids. If glutamic acid is classified a dispensable amino acid, it *necessitates* providing other non-essential amino acids in the diet. In the present study using 11 amino acids, glutamic acid must be considered an essential amino acid, although the possibility that it could be replaced with other amino acids considered non-essential is not discounted.

Difficulty was experienced in demonstrating the non-essentiality of tyrosine in the presence of increased levels of phenylalanine. The recent work of Armstrong ('55) which indicated very inefficient conversion of phenylalanine to tyrosine in the rat suggests that even higher levels of phenylalanine than have been tried in the present study would permit normal egg production in all hens. On the other hand, Benton et al. ('56) have shown that phenylalanine at high levels (approached in this study when the DL form was used) acts as an antagonist towards other amino acids. Thus, the combination of an inefficient conversion and amino acid antagonism exhibited by phenylalanine may explain the present observation.

The remarkable uniformity in the egg production pause resulting from the omission of any one essential amino acid (glutamic acid was not included in this experiment; however, see table 3) is similar to the uniform daily loss in body weight exhibited by chicks when fed diets lacking one of the essential amino acids (Almquist, '47). This suggests that as for growth, the amino acid requirement for egg protein synthesis is an aggregate one in that all amino acids must be present simultaneously.

During the 5-day experimental period, hens on the control diet tended to lay slightly more eggs (table 4) than those

on a diet from which one of the essential amino acids were removed; the difference, however, was not statistically significant. The remarkable uniformity in results on either the complete or incomplete diets supports the adequacy of short-period studies with free amino acids diets. The fact that hens are able to lay very few eggs on a deficient diet suggests that they have small protein stores which became rapidly depleted on a deficient diet. Egg formation thus offers a unique opportunity for studying protein synthesis and amino acid turnover *in vivo*; it is hoped that knowledge of the essential amino acids required for this process as well as the availability of a free amino acid diet will enhance such studies in the future.

#### SUMMARY

The classification of amino acids according to their essentiality for the laying hen has been studied by use of a free amino acid diet. Arginine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were found to be essential for egg production. With the exception of glutamic acid, omission of any one of the above amino acids resulted in immediate disruption of feed consumption and a 10-day pause in production when such incomplete diets were force-fed for only 5 days. Although feed consumption was not affected by the omission of glutamic acid, normal egg production could not be maintained. Tyrosine could be replaced by phenylalanine only with difficulty. The hen does not require glycine for egg production. This is in contrast to the need of the growing chick for this amino acid.

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# FURTHER EVIDENCE ON THE REQUIREMENT OF THE CHICK FOR UNIDENTIFIED MINERALS<sup>1</sup>

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Morrison, Scott and Norris ('55) discovered that, when a mixture of materials containing unidentified chick growth factors is fed to chicks, a portion of the growth response obtained from them is due to a mineral constituent(s) of the mixture not previously reported to be required by animals. At approximately the same time, Dannenburg, Reid, Rozacky and Couch ('55) found that the ash of corn distillers' dried solubles promoted increased growth in chicks. The studies presented in this report confirm and extend the original observations made by these groups of investigators and indicate that the mineral(s) involved is concerned in bone formation.

## EXPERIMENTAL

The purified basal diet used in most experiments reported herein contained the following ingredients per 100 gm: glucose,<sup>2</sup> 61.30 gm; purified isolated soybean protein,<sup>3</sup> 25.57 gm; hydrogenated fat,<sup>4</sup> 3 gm; ground cellulose,<sup>5</sup> 3 gm; DL-methio-

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<sup>2</sup> Corden Co.

<sup>3</sup> Duckett Amino Protein C-1.

<sup>4</sup> Hy-Less, Leavitt Bros.

<sup>5</sup> F. W. Hoar, The F. W. Hoar Company, Piquette, N. H.

nine, 0.7 gm; glycine, 0.3 gm;  $\text{CaHPO}_4$ , 2.151 gm;  $\text{CaCO}_3$ , 1.492 gm;  $\text{KH}_2\text{PO}_4$ , 0.867 gm;  $\text{NaCl}$ , 0.6 gm;  $\text{MgSO}_4$ , 0.25 gm;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0333 gm;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.0333 gm;  $\text{KI}$ , 0.26 mg;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1.67 mg;  $\text{ZnCl}_2$ , 1.0 mg;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.17 mg;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.83 mg; choline  $\text{Cl}$ , 0.15 gm; inositol, 25.0 mg; niacin, 5.0 mg; calcium pantothenate, 2.0 mg;  $\alpha$ -tocopheryl acetate, 2.0 mg; thiamine  $\text{HCl}$ , 1.0 mg; riboflavin, 1.0 mg; pyridoxine  $\text{HCl}$ , 0.45 mg; folic acid, 0.40 mg; menadione, 0.05 mg; biotin, 0.02 mg; vitamin  $\text{B}_{12}$ , 2.0  $\mu\text{g}$ ; vitamin A, 500 I.U.; vitamin  $\text{D}_3$ , 37.5 I.C.U. The mineral compounds in the mineral mixture used in the first few experiments were reagent grade except the dicalcium phosphate ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ ) and the calcium carbonate, which were U.S.P. grade. The quantity of dicalcium phosphate used in the mineral mixture was adjusted for water of hydration. In all subsequent experiments reagent grade minerals were used exclusively. The isolated soybean protein was purified by repeated washings at its isoelectric point of pH 4.6. The supernatant liquid was decanted after each washing. The mineral content of the soybean protein was reduced from 2.8 to 1.3% by the purification process. In a few studies purified casein plus the necessary essential amino acids, or a mixture of purified casein and purified isolated soybean protein, was used as the source of protein in the basal diet. The casein was purified by repeatedly washing in a brine solution at pH 2.5, followed by several washings at the isoelectric point of pH 4.7. The basal diet contained, by calculation, adequate or excess quantities of the nutrients known to be required by the chick.

All additions to the basal diet were made in such a way as to maintain the protein level constant at 20.5%. In experiments in which the minerals of the unidentified growth factor supplements were added to the basal diet, substitution was made at the expense of glucose. The minerals were obtained by burning off much of the organic matter of the crude materials in an evaporating dish, followed by incineration in a

muffle furnace at 525°C. for 4 hours. The inorganic residue then was cooled and brought to pH 7 with glacial acetic acid.

Either New Hampshire  $\times$  Barred Plymouth Rock or White Plymouth Rock chicks of hens fed diets which produce chicks deficient in unidentified growth factors, according to Waibel, Morrison and Norris ('55), were used in most studies. In a few experiments, however, White Plymouth Rock chicks obtained from a commercial hatchery were used. The chicks were brooded in electrically heated, galvanized metal battery brooders with raised screen floors, and feed was supplied *ad libitum*. Distilled or demineralized water was supplied in metal watering pans sprayed with plastic paint, except in several of the experiments conducted at the beginning of the investigation. The chicks were identified by numbered wing bands at the start of the experiment and weighed individually at weekly intervals thereafter until they were 4 weeks of age, at which time most of the experiments were terminated. The quantity of feed consumed by the chicks was recorded.

#### RESULTS AND DISCUSSION

In studies conducted to prove the adequacy of the basal diet in nutrients known to be necessary, it was found that the addition of linoleic acid, increased quantities of all known essential amino acids, or all known essential vitamins, had no influence on growth. The addition to the basal diet of increments of reagent grade magnesium sulfate failed to increase the rate of growth. In several experiments, it was found that increased quantities of sodium or potassium, either alone or in combination, as the chloride or sulfate salts, did not improve growth. Alteration of the calcium and phosphorus content of the basal diet by the addition of 1% of dicalcium phosphate failed to increase chick growth. Additional chlorine had no effect on growth. The addition to the basal diet of 50 or 100% more of the essential trace elements (iron, copper, manganese, cobalt, iodine, zinc), essential major elements (calcium, phosphorus, magnesium, potassium, sodium, chlorine) or the complete mineral mixture did not appreciably

increase growth. Although molybdenum has been shown to be an integral part of certain enzyme systems, the results of two experiments showed that the addition of molybdenum to the basal diet failed to promote a growth increase. Edwards et al. ('55) were also unable to obtain any growth response from the addition of molybdenum to a purified chick diet similar to the diets used in the present studies. Inasmuch as Almquist and Meechi ('40) had previously reported growth stimulation in chicks from sodium acetate, and because glacial acetic acid was used to neutralize the ignited materials, 0.20% sodium acetate was added to the basal diet, but this failed to improve growth. Since Machlin ('55) found that under certain conditions sulfate *per se* stimulated the growth of chicks, 0.17% sulfate was added to the basal diet, but it failed to influence the rate of gain.

Although it was not possible to increase chick growth by increasing the amounts of the essential nutrients in the basal diet, it was found, in confirmation of previous work from this laboratory, that the addition to the basal diet of 18% of a mixture of 5 unidentified growth factor supplements consisting of 6 parts of corn distillers' dried solubles, 3 parts of fish solubles, 3 parts of dried whey product, 3 parts of forage juice, and 3 parts of penicillin mycelium meal elicited a highly significant ( $P < 0.01$  by analysis of variance) growth response at 4 weeks of age. The combined results of a number of experiments are given in table 1. Chicks fed the basal diet supplemented with 5 sources of unidentified growth factors grew for the most part as rapidly, and frequently more rapidly, than those fed a good quality commercial chick ration.

The results further showed that a considerable portion of the growth response produced by the addition to the basal diet of sources of unidentified growth factors is due to an unknown mineral nutrient or nutrients contained in them, since a highly significant ( $P < 0.01$ ) growth response was also obtained from the inorganic portion of the crude materials. In addition to stimulating growth, the mixture of 5 unidenti-

fied growth factor supplements, or its ash, produced a highly significant ( $P < 0.01$ ) increase in efficiency of feed utilization.

In other experiments, the results of which are also presented in table 1, the addition to the basal diet of 6% of a composite sample of corn distillers' dried solubles, or the minerals supplied by 6% distillers' dried solubles, promoted highly significant ( $P < 0.01$ ) growth increases at 4 weeks of age. The efficiency of feed utilization was also increased.

TABLE 1

*Evidence of an unidentified mineral(s) required for chick growth*

TREATMENT	AV. WT. 4 WKS.	GAIN OVER BASAL	GAIN/ FEED
	gm	%	gm
A. Response to UFS <sup>1</sup> and the ash of UFS			
Basal	306 (13) <sup>2</sup>	.	0.493
+ ash UFS <sup>3</sup>	350 ( 8)	24.2	0.571
+ UFS	416 ( 8)	35.9	0.595
B. Response to DDS <sup>4</sup> and the ash of DDS			
Basal	298 (12)	.	0.500
+ ash 6% DDS <sup>5</sup>	355 ( 9)	19.1	0.543
+ 6% DDS	368 ( 9)	23.5	0.562

<sup>1</sup>UFS—mixture of unidentified factor supplements.

<sup>2</sup>Number of lots of approximately 20 chicks each.

<sup>3</sup>Ash amounted to approximately 2% of total diet when fed at a level equivalent to 6% distillers' dried solubles, 3% fish solubles, 3% dried whey product, 3% forage juice and 5% penicillin mycelium meal.

<sup>4</sup>DDS—distillers' dried solubles.

<sup>5</sup>Ash amounted to approximately 10% of total diet when fed at a level equivalent to 6% distillers' dried solubles.

The experiments on the ash of distillers' dried solubles were not conducted simultaneously in all instances with those on the ash of the mixture of unidentified factor supplements. Although an apparent difference in percentage growth responses was observed, this is not believed to be significant, since in three comparable experiments the average growth (358 gm) obtained at 4 weeks with the ash of the mixture of unidentified factor supplements was no greater than that (375 gm) obtained with the ash of distillers' dried solubles.



A highly significant difference ( $P < 0.01$ ) between the growth response promoted by the 5 unidentified factor sources and that obtained from the ash of these materials was observed in the experimental work summarized in table 1. In later work, the results of which are presented in table 2, larger quantities of ash than those usually fed, prepared either from 5 unidentified growth factor supplements or 3 unidentified growth factor supplements (6 parts of corn distillers' dried

TABLE 2

*Differentiation of unidentified organic and inorganic chick growth factors*

TREATMENT	AV. WT. 4 WKS. gm	GAIN OVER BASAL %
<i>Experiments with UFS<sup>1</sup> ash</i>		
Basal	287 (4) <sup>2</sup>	...
+ 1.20 or 2.0% ash <sup>3</sup>	323 (4)	14.5
+ 1.80 or 3.0% ash	323 (4)	14.5
+ 3 UFS	382 (2)	38.2
<i>Experiments with DDS<sup>4</sup> ash</i>		
Basal	305 (4)	...
+ 0.56% ash	352 (3)	17.7
+ 0.75 or 0.85% ash	347 (3)	15.8
+ 5 UFS	408 (3)	38.9

<sup>1</sup> UFS = mixture unidentified factor supplements.

<sup>2</sup> Number of lots of approximately 20 chicks each.

<sup>3</sup> Smaller quantity of ash from mixture of 3 UFS; larger quantity from mixture of 5 UFS.

<sup>4</sup> DDS = distillers' dried solubles.

solubles, 3 parts of fish solubles and 3 parts of dried whey product) did not further stimulate growth. On the other hand, the intact unidentified growth factor supplements significantly ( $P < 0.01$ ) increased the growth of chicks over that obtained with the ash. Larger quantities of the ash of distillers' dried solubles also failed to increase chick growth above the responses obtained from the amount usually supplied. In contrast the growth of the chicks supplied the 5 unidentified growth factors supplements was significantly ( $P < 0.01$ ) greater than that obtained with the ash of the distiller's

dried solubles. Therefore, the results showed that an unidentified organic factor(s), as well as an unidentified inorganic essential(s), is required for maximum chick growth.

Although our data suggest the existence of an unidentified organic growth factor(s) in distillers' dried solubles, there was no statistically significant difference between the growth response obtained from the intact solubles and that obtained from the ash. However, our failure to show a significant response from the organic factor in distillers' dried solubles may have been due to a deficiency of the organic factor(s) present in the other materials in the mixture of 5 unidentified factor supplements. Novak and Hauge ('48a, b) and Dannenburg et al. ('55) have obtained evidence that corn distillers' dried solubles contains an unidentified organic factor(s).

That the distribution of the unidentified inorganic essential(s) may be widespread is suggested by the fact that chick growth responses from diets believed to be adequate in the known nutrients have been obtained from the minerals of dried whey (Couch et al., '55), fish meal (Tamimie, '55), feather meal (Menge et al., '56), and gelatin (Benton et al., '55). The minerals of fish solubles were also found to promote an increase in growth at this laboratory.

Carcass analysis studies showed that the additional gain observed in chicks fed the basal diet supplemented with the ash of 5 unidentified growth factor supplements was not due to increased water retention. The percentages of moisture, protein, ether extract, and ash in the carcasses of chicks fed the basal diet were found to be 71.93, 17.61, 7.32 and 2.80 respectively. The corresponding values for the chicks fed the basal diet plus the ash of 5 unidentified factor sources were 73.18, 17.76, 5.83 and 2.77% respectively.

Highly depleted chicks fed the basal diet exhibited a bone malformation, characterized by enlargement and elongation of the intertarsal (hock) joint. In repeated experiments, it was observed that an average of 26% of the chicks fed the basal diet exhibited the leg bone malformation, while the average incidence of the syndrome in chicks receiving the basal

diet plus 5 unidentified growth factor supplements, or the ash of these 5 supplements, was only 2% and 7% respectively.

In further studies the percentage ash (45.07%) in the dry fat-free tibiotarsae of chicks fed the basal diet was found to be significantly lower ( $P < 0.02$ ) than that (47.30%) of chicks of equal weight fed the basal diet plus the ash of 5 unidentified growth factor supplements. Although the tibiotarsae of chicks fed the basal diet were slightly shorter than those of chicks of equal weight fed the basal diet plus the ash of 5 unidentified growth factor supplements, the difference was not found to be statistically significant. The breaking strength (5.99 kg) of the tibiotarsae of chicks receiving the ash was greater ( $P < 0.10$ ) than that (5.43 kg) of chicks receiving the basal diet. These results are similar to those reported by Caskey, Gallup and Norris ('39) on the effect of manganese deficiency on bone development. However, in the present studies, additional manganese had no effect upon growth or incidence of leg malformation, and slipped tendon, or perosis, has been only rarely observed.

Studies on the blood of chicks receiving the basal diet or the basal diet supplemented with the ash of 5 unidentified growth factor supplements showed that the ash had no effect on the amount of hemoglobin, volume of red cells or red cell count. At 4 weeks of age, the results of these determinations on the blood of chicks fed the basal diet were 9.5 gm%, 26% and 2.06 million/mm<sup>3</sup>, respectively. The corresponding values for chicks fed the basal diet plus the ash of 5 unidentified growth factor supplements were 9.8 gm%, 26% and 2.13 million/mm<sup>3</sup>, respectively.

Attempts to increase chick growth by feeding a mixture of reagent grade minerals which was prepared in the proportions indicated by the spectrographic analysis of the ash of distillers' dried solubles failed to promote increased growth, in contrast to the finding of Reid, Rozacky and Couch ('55). The spectrographic analysis of the ash of distillers' dried solubles reported by Couch and associates ('55) and that obtained through the courtesy of James McGinnis of the State

College of Washington were used in formulating the mixture of reagent grade minerals. The reconstituted mineral mixture contributed the following elements to the diet in parts per million: Al, 3.0; B, 1.8; Ba, 0.48; Ca, 60.0; Cr, 0.036; Cu, 9.6; Fe, 60.0; Pb, 0.12; Mg, 120.0; Mn, 3.0; Mo, 0.13; Ni, 0.12; P, 180.0; K, 180.0; Si, 60.0; Ag, 0.024; Na, 180.0; Sr, 120; Ti, 0.24; V, 0.12; Zn, 3.0. The results of growth studies conducted on this phase of the problem are given in table 3. The discrepancy in the results obtained by the two groups of workers may pos-

TABLE 3  
*Effect of synthetic mineral mixtures on chick growth*

TREATMENT	AV. WT. 4 WKS.	GAIN OVER BASAL
	gm	%
<i>Response to reconstituted mineral mixture</i>		
Basal	297 (4) <sup>1</sup>	...
+ ash 5 UFS <sup>2</sup>	333 (4)	12.1
+ mineral mixture <sup>3</sup>	295 (4)	0
<i>Response to Hoagland and Snyder's mineral solution</i>		
Basal	280 (5)	...
+ ash 5 UFS	336 (5)	23.1
+ mineral solution <sup>4</sup>	314 (5)	14.0

<sup>1</sup> Number of lots of approximately 20 chicks each.

<sup>2</sup> UFS = mixture unidentified factor supplements.

<sup>3</sup> Formulated on the basis of spectrographic analyses.

<sup>4</sup> Added to basal diet at a level of 150 mg/kg.

sibly be explained by differences in reagent grade mineral salts used in making up the reconstituted mineral mixtures.

Although a mineral mixture formulated on the basis of spectrographic analyses did not increase growth, the data in table 3 show that the complex mineral solution of Hoagland and Snyder (33) was partially as effective in increasing chick growth as the mineral portion of the mixture of 5 unidentified growth factor supplements.

In further studies designed to determine the biologically active component(s) in the ash of the 5 unidentified growth factor supplements, the addition of salts of aluminum, arsenic,

barium, beryllium, bismuth, boron, bromine, cadmium, cerium, cesium, chromium, cobalt, fluorine, indium, iridium, lead, lithium, mercury, nickel, platinum, rhodium, rubidium, ruthenium, selenium, silicon, silver, strontium, tantalum, tellurium, thorium, tin, titanium, tungsten, vanadium, yttrium or zirconium, did not influence growth at the levels used. These included the mineral elements not known to be required in animal nutrition which are present in the salt solution of

TABLE 4

*Results of studies on isolation of unidentified mineral(s) in the ash*

TREATMENT	AV. WT. 4 WKS.	GAIN OVER BASAL
	gm	%
<i>Response to water insoluble fraction</i>		
Basal	285 <sup>1</sup>	...
+ ash 5 UFS <sup>2</sup>	324	13.7
+ water soluble portion	304	6.7
+ water insoluble portion	333	16.8
<i>Response to cation exchanger elutriant</i>		
Basal	321	...
+ ash 3 PFS <sup>3</sup>	347	8.1
+ IR-120 <sup>4</sup> effluent of ash	311	- 3.1
+ IR-120 elutriant of ash	351	9.3

<sup>1</sup> Average duplicate lots of 20 chicks per treatment.

<sup>2</sup> UFS = mixture unidentified factor supplements.

<sup>3</sup> Distillers' dried solubles, 6 parts; fish solubles, 3 parts; dried whey product, 3 parts.

<sup>4</sup> Amberlite resin IR-120, Rohm and Haas Company.

Hoagland and Snyder ('33). Many of the above elements have also been tested for biological activity by Dannenburg et al. ('55) and Reid, Rozaeky and Couch ('55) with results similar to those obtained by us.

In fractionation studies, the results of which are given in table 4, evidence was obtained that the active component(s) in the ash of the mixture of 5 unidentified growth factor supplements is present in that portion insoluble in boiling water. The ash was treated with 5 volumes of boiling water for 30 minutes, filtered, and the insoluble residue again extracted

with an additional 5 volumes of boiling water for 30 minutes. Under these conditions, it was found that approximately 35% of the ash was water soluble. In a further experiment, the ash was prepared by a wet-ashing procedure, wherein the crude unidentified growth factor supplements were digested with concentrated nitric acid. The results indicated that ash prepared by this procedure was fully as active biologically as ash prepared by the usual incineration procedure.

In an additional study, the ash was prepared by the wet-ashing procedure, dissolved in nitric acid at pH 2.35, and passed through a strong cation exchanger<sup>6</sup> in the hydrogen form. The cations retained on the column were eluted with 10% nitric acid. The elutrient and effluent were concentrated, neutralized, and added to the basal diet. The results of the experiment are given in table 4. They indicated that the growth-promoting effect of the ash is due to a cation(s) since the increased growth obtained with the cationic fraction was highly significant ( $P < 0.01$ ).

Studies have also been conducted to determine if the effect of the unidentified minerals in the ash of unidentified growth factor supplements is a direct one or mediated through the microflora of the intestinal tract. The results of the work revealed no consistent effect of the ash on the numbers of aerobes, anaerobes, coliforms, lactobacilli, streptococci or clostridia in either the entire intestinal tract or sections of it. The ash, furthermore, exerted no effect on the pH of the intestinal tract. The results suggested, therefore, that the primary effect of the ash is exerted directly upon the tissues of the chick rather than indirectly through effects on the intestinal microflora.

#### SUMMARY

In further work on unidentified chick growth factors results were obtained which indicated that the basal diets supplied the chicks were adequate in amino acids, known vitamins and

<sup>6</sup>Amberlite MB 3, Rohm and Haas Company.

minerals, previously reported to be needed by animals. No evidence of an imbalance was obtained in the studies on required minerals. As a consequence it has been found, in confirmation of previous reports by this laboratory, that when a mixture of unidentified growth factor supplements is fed to chicks the growth response which is observed is due to the presence in the materials of both unidentified organic and inorganic constituents. The results of the investigation showed, therefore, that a mineral or minerals not hitherto considered to be essential in the nutrition of the chick is present in the mineral portion or ash of certain crude feedstuffs. The unidentified mineral(s) was found to be involved in bone formation. Evidence was also obtained which indicated that the unknown mineral nutrient(s) is present in the boiling water-insoluble fraction of the ash of the mixture of unidentified factor supplements, and that it is cationic in acid solution. No consistent effect of the ash on the intestinal microflora of the chicks or the pH of the intestinal contents was observed.

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# NUTRITIVE VALUE OF PROTEIN AND TUMOR-HOST RELATIONSHIP IN THE RAT<sup>1</sup>

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Previous studies have emphasized the variable responses of different types of tumors to the diet of the host, one of the most interesting responses being associated with the kind and quantity of dietary protein (Mider, '51; Le Page et al, '52; Tannebaum and Silverstone, '53; Babson, '54; Babson and Winnick, '54; Greenlees and Le Page, '55). These authors and others have presented data that can be interpreted to mean that some tumors deplete the body of labile protein stores more than others, the depleting effect of a growing neoplasm varying with the size and nature of the tumor. Sherman et al. ('50) suggested, for example, that the Walker carcinoma 256 in rats depleted these stores after the tumor had become 10% of the total body weight.

Recent reports from our laboratories have emphasized the effect of dietary protein upon tumor-host interrelationships and upon the response of tumor and normal tissues to chemotherapy with the ethylenephosphoramides (Allison et al., '54, '55, '56; McCoy et al., '56). Studies involving a transplanted sarcoma in the rat, for example, demonstrated that although the growth of this neoplasm was not easily affected by dietary protein, it developed most rapidly in animals fed a semi-synthetic diet containing 12% casein. Supplementing

<sup>1</sup> This paper is being published with the permission of the American Cancer Society upon the basis of a grant from the National Cancer Institute, U. S. Department of Health, Education and Welfare.

with methionine or still better with methionine plus guanidoacetic acid favored the growth of normal tissues and, if the growth of these tissues was maximum, the development of the tumor was depressed. The favorable response to chemotherapy was also maximum under these conditions of optimum development of normal tissues (Allison et al., '55; Allison, '56). The Walker carcinoma 256 was another tumor that grew rapidly at the expense of normal tissues and was resistant to ethylenephosphoramidate therapy. The Flexner-Jobling carcinoma, on the other hand, under the conditions of our experiments was most susceptible to this type of therapy, the majority of the tumors completely regressing (Crossley et al., '53) and they seemed to be affected more than the other two tumors by the kind and amount of protein in the diet (Babson, '54).

The following experiments were done, therefore, to further characterize these three tumors according to the effect of dietary protein upon their development in the host, hoping thereby to derive a better basis for evaluating the effects of various drugs and diets upon protein metabolism in the tumor and in the host.

#### METHODS

The semi-synthetic diet and the methods of transplanting and measuring the development and growth of the tumor were described previously (Allison et al., '54). Three different tumors were used in these studies, the Sarcoma R-1, the Walker carcinoma 256, each transplanted into male Wistar rats, and the Flexner-Jobling carcinoma transplanted into male Sprague-Dawley rats. Ten rats containing one type of tumor were placed on each diet at the time of transplantation. The groups were pair-fed the diet containing different proteins, wheat gluten, casein, beef, and egg albumin. These proteins were of the same type or source reported in previous studies from our laboratory (Allison, '55), the protein forming 12% of the dry diet. The effects on the growth of the tumor and body of supplementing casein with 0.7% methionine

were studied also, using techniques described previously (Allison et al., '54). Similarly groups were fed the 12% casein diet supplemented with mixtures of 0.7% methionine plus 0.7% guanidoacetic acid, 0.7% methionine plus 1.28% glycine, and 0.7% methionine plus 1.95% ammonium citrate (Allison et al., '56). The tumors were allowed to develop to average approximately 17 to 20 gm in rats fed the egg albumin, tumor sizes which approached lethal proportions in animals fed the 12% casein diet. To develop tumors of this size required three weeks in rats bearing the Sarcoma R-1, 11 days in rats with the Walker carcinoma 256 and 28 days in the Sprague-Dawley rats with the Flexner-Jobling carcinoma. Tumors one gram or less in size at the end of the experimental period were considered as not taking.

#### RESULTS

The protein efficiencies (grams gained per gram of nitrogen intake) for normal and sarcoma-bearing rats are illustrated in figure 1. The white bars record the protein efficiencies for groups of normal rats, pair-fed isocaloric diets containing equivalent amounts of wheat gluten, casein, beef, or egg albumin. These protein efficiencies agree well with those determined previously in rats and they illustrate the relative nutritive values of these proteins for growth of the soft tissues of most animals (Allison, '55). The black bars record the gain in weight of the body per gram of nitrogen intake in sarcoma-bearing rats. This gain, calculated by subtracting the weight of the tumor from the total weight of the tumor-bearing rat, was of the same order as in normal rats but less in magnitude, a reduction that is to be expected because of the utilization of nitrogen by the growing sarcoma. The presence of the sarcoma increased the protein efficiencies of the tumor-bearing animals much above the controls as illustrated by the bars with slanted lines.

The weight of the sarcoma, 21 days after transplantation, was slightly but significantly smaller in animals fed the diet containing egg albumin than in animals fed other proteins

in the diet (table 1). These results are in agreement with other data obtained using this sarcoma, data which demonstrated the sarcoma developed slower in animals where normal tissues developed maximally (Allison et al., '56). The rate of growth of the body with respect to tumor was lowest

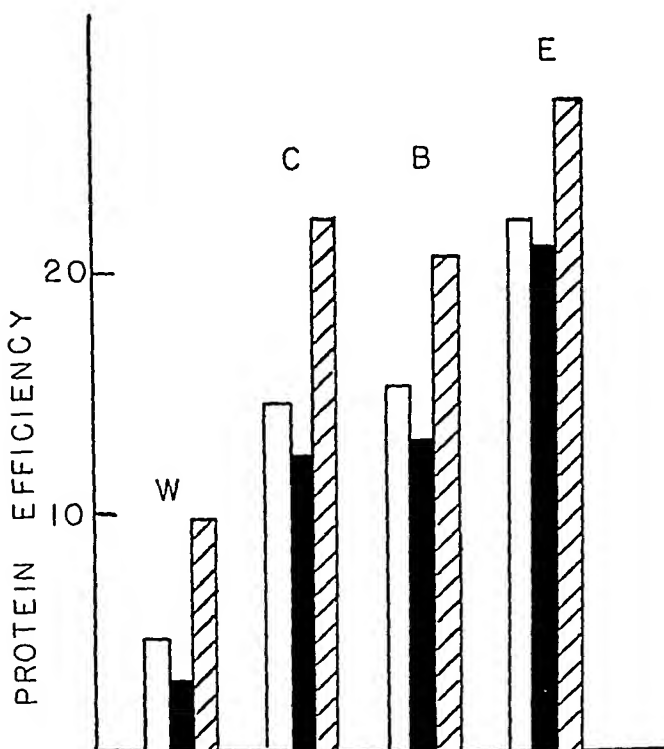


Fig. 1 Protein efficiencies (grams gained in weight per gram nitrogen intake) in normal rats (white bars), body of sarcoma-bearing rats (black bars), and whole sarcoma-bearing rats (bars with slanted lines), the rats being pair fed different proteins—wheat gluten, W; casein, C; beef, B; and egg albumin, E.

(0.5) in animals fed wheat gluten, intermediate (1.6) in those fed casein or beef and highest (3.4) in sarcoma-bearing rats fed egg albumin (table 1). Previous experience has demonstrated that the welfare of the animal, together with resistance to therapeutic stress, increased as the ratio increased (Allison et al., '54, '55, '56).

TABLE 1

Protein diet utilized on groups of tumor-bearing rats, pair fed so that the nitrogen and caloric intakes were approximately constant for each experiment. Gain in body weight over the experimental period was calculated as equal to total weight minus the weight of the tumor

Diet	MUCOMA (21 DAYS)				WALKER CARCINOMA 256 (11 DAYS)				F-J CARCINOMA (28 DAYS)			
	No. animals	Tumor	$\Delta$ body tumor	gms	No. animals	Tumor	$\Delta$ body tumor	gms	No. animals	Tumor	$\Delta$ body tumor	gms
Wheat gluten	9	24.7 $\pm$ 3.6	0.5 $\pm$ 0.1	8	18.3 $\pm$ 1.3	0.1 $\pm$ 0.2	9	5.3 $\pm$ 1.6	9	5.3 $\pm$ 1.6	1.1 $\pm$ 1.4	
Caseln	8	20.1 $\pm$ 4.0	1.6 $\pm$ 0.3	8	23.4 $\pm$ 3.0	1.7 $\pm$ 0.3	6	11.3 $\pm$ 2.2	6	11.3 $\pm$ 2.2	5.0 $\pm$ 1.2	
Peanut	9	28.2 $\pm$ 2.1	1.7 $\pm$ 0.2	9	24.9 $\pm$ 1.0	1.4 $\pm$ 0.3	7	18.6 $\pm$ 4.0	7	18.6 $\pm$ 4.0	3.1 $\pm$ 0.8	
Peanut albumin	9	20.0 $\pm$ 1.0	3.4 $\pm$ 0.3	9	17.7 $\pm$ 1.5	2.5 $\pm$ 0.2	7	20.0 $\pm$ 3.2	7	20.0 $\pm$ 3.2	4.4 $\pm$ 2.1	

Feeding these same diets to rats bearing the Walker carcinoma 256 resulted in tumor growth and body weight gains similar to those found for the sarcoma-bearing animals (table 1). The Walker tumor, for example, was slightly but significantly smaller in rats fed albumin than in those fed casein. The rate of gain of the body with respect to the tumor was very low (0.1) in animals fed wheat gluten, was intermediate in animals fed casein or beef (1.4 to 1.7) and was highest (2.5) in tumor-bearing rats fed egg albumin.

The data recorded in table 1 demonstrate, on the other hand, that the Flexner-Jobling carcinoma responded as did the body to the type of protein in the diet, growing poorly in rats fed wheat gluten and most rapidly in animals fed egg albumin. The results suggest that this carcinoma did not grow at the expense of normal tissues as much as the other two neoplasms, a suggestion which is supported by the high body weight gain/tumor ratios in animals fed beef, casein, and egg proteins. The larger carcinoma in the animals fed egg albumin may have had a sufficient depleting effect to keep the ratios from increasing above those found for casein and beef, an increase that would be expected on the basis of relative protein efficiencies. These results using the three types of tumors demonstrate that, under the experimental conditions used here, the Flexner-Jobling carcinoma is affected more by the protein in the diet than are the sarcoma or the Walker neoplasm. Possibly tumors vary as do different normal tissues in their interrelationship with the body metabolic pools. In animals fed a protein-free diet, for example, some tissue proteins are depleted more than others and some are not depleted at all (Allison, '55). It may be significant that the ethylenephosphoramides, which seem to have some inhibiting effect upon protein anabolism (Allison et al., '54; McCoy et al., '56), are much more effective in reducing the growth and causing the regression of the Flexner-Jobling carcinoma than the other two types of tumors.

Increase in liver protein has been used as a measure of nutritive value of the dietary protein (Addis et al., '36; Kos-

terlitz and Campbell, '45-'46; Harrison and Long, '45). Total liver protein was lowest in normal or sarcoma-bearing animals fed wheat gluten and highest in those fed egg albumin, data illustrated in figure 2. The relatively higher liver protein, however, in the tumor-bearing animals than in the controls has been observed by others (Yeakel and Tobias, '51; Le Page et al., '52; Babson, '54). Such an increase in liver

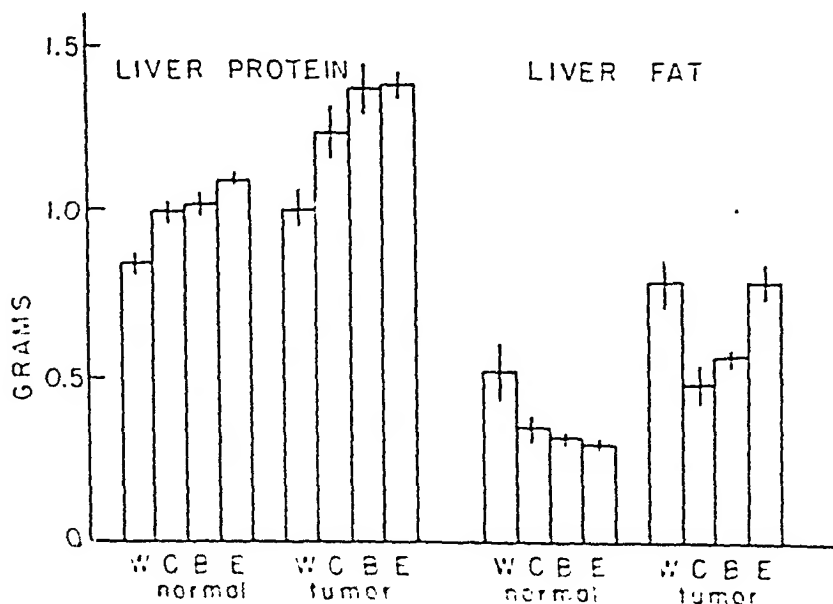


Fig 2 Total liver protein and fat in normal and sarcoma-bearing rats fed wheat gluten, W; casein, C; beef, B; and egg albumin, E. The standard errors of the averages are illustrated by the lines through the bars.

protein is associated with the high catabolic activity of the tumor-bearing animal when the tumor has reached depleting and lethal proportions (Allison et al., '56). The suggestion has been made that liver nitrogen increases at a time when this organ is involved in coping with split products from large necrotic tumors (Sherman et al., '50). Greenlees and Le Page ('55) did not observe enlarged livers in their studies since "the tumors never constituted more than 7 per cent of total body weight, and toxic effects from necrotic tissue



should be negligible." The suggestion has been made also that the sarcoma growing at the expense of normal tissues, created an overall amino acid imbalance in the animal (Allison, '55), thereby increasing both total liver protein and liver fat.

The higher liver fat in the normal animals fed wheat gluten is a common response to this deficient diet. The relatively higher liver fat in the tumor-bearing animals possibly is correlated with the formation and transport of lipid and lipoprotein in the presence of the large tumor. A marked lipemia developed in the sarcoma-bearing animals fed 12% casein, a diet which seemed to be optimum for the development of this tumor (see also Mider, '51). The addition of methionine to the diet reduced the lipemia and also slowed the development of the tumor (Allison et al., '54, '56).

Data have been presented to show that supplementation of the casein diet with an optimum amount of DL-methionine, or still better with a mixture of DL-methionine and guanidoacetic acid, improved the growth of normal tissues in the sarcoma-bearing rats (Allison et al., '56). Under these conditions, as with the improved growth of normal tissues in animals fed egg albumin, the sarcoma developed most slowly. Similarly the Walker carcinoma 256 developed more slowly and the normal tissues grew more rapidly in tumor-bearing animals fed casein supplemented with 0.7% DL-methionine than in rats fed unsupplemented casein. Adding glycine or ammonium citrate in equivalent amounts of nitrogen did not change significantly the supplementing effect of methionine. Adding 0.7% guanidoacetic acid with the 0.7% DL-methionine, however, reduced the size of the tumor significantly and increased the body gain/tumor ratio markedly (see figure 3). This effect of a mixture of methionine and guanidoacetic acid was most evident when the tumor became sufficiently large to produce a marked depleting effect, at a time when the catabolic activity as measured by urea nitrogen excretion was relatively high (see Allison et al., '56). The urea nitrogen excretion, for example, in these animals 11

days after transplantation of the tumor, when the excretion of urea would have been relatively low in a depleted rat (Allison et al., '56) was as follows: tumor-bearing rats fed 12% casein, 21 mg.; animals fed casein supplemented with methionine, 19.5 mg; those fed casein supplemented with methionine plus glycine, 30 mg; those fed methionine plus

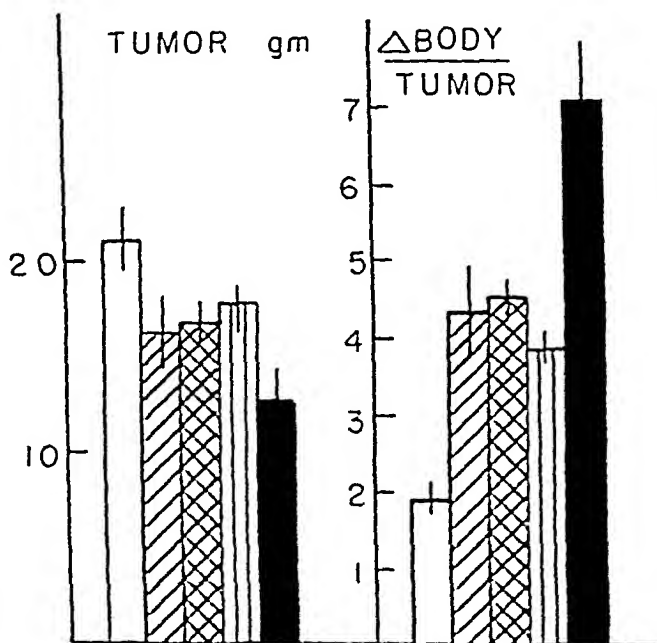


Fig. 3. The effect of supplementation upon the development of the Walker carcinoma 256 and upon the increase in weight of the body with respect to the tumor. White bars record average data for tumor-bearing rats fed diet containing 12% casein. Supplementation with methionine, bars with slanted lines, with methionine plus glycine, bars with crossed lines; methionine plus ammonium citrate, bars with vertical lines; methionine plus guanidoacetic acid, black bars.

ammonium citrate, 31 mg; and sarcoma-bearing rats fed methionine plus guanidoacetic acid, 11 mg urea nitrogen/rat/day. As in other experiments this reduction in catabolic activity (as measured by urea nitrogen) in the presence of a mixture of methionine and guanidoacetic acid was not observed until the tumor was large and, thereby, at a time

when a marked stress was being placed upon the animal. No reduction in the growth of the Flexner-Jobling carcinoma nor increase in the already relatively large body weight gain/tumor ratio was observed when rats were fed casein supplemented with either methionine or a mixture of methionine and guanidoacetic acid. Rather, supplementation of this type tended to improve the growth of the tumor slightly, as was the case in Flexner-Jobling carcinoma-bearing animals fed a protein of high nutritive value such as egg albumin.

#### SUMMARY

The effects of dietary protein upon the development of three types of transplanted tumors were studied in the rat. A sarcoma developed at essentially the same rate in rats fed diets containing wheat gluten, casein or beef but was slightly depressed in animals fed egg albumin. The bodies of the rats, however, developed according to the nutritive value of the protein, growing poorly in animals fed wheat gluten and at a maximum in those fed egg albumin. During the terminal stages the sarcoma increased catabolism and depleted normal tissues but increased relatively the liver protein and liver fat above pair fed controls. Similar effects on tumor and body growth were observed in rats bearing the Walker carcinoma 256. The development of the Flexner-Jobling carcinoma, on the other hand, was very poor in animals fed wheat gluten and maximum in rats fed egg albumin, a response that was more like the body tissues. Supplementation of the casein diet with methionine or methionine plus guanidoacetic acid had beneficial effects upon the development of normal tissues in the presence of the sarcoma or Walker carcinoma 256, but was much less beneficial in the presence of the Flexner-Jobling carcinoma. The suggestion was made that tumors may vary as do normal tissues in their interrelationships to body metabolic pools, some tissue proteins being depleted more than others, some not at all in the presence of inadequate amino acid intake. A possible correlation between protein anabolic, catabolic influence of these

tumors and the effects of ethylenephosphoramides was also suggested.

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Nominations are solicited for the 1957 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this honor.

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University of Colorado School of Medicine  
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The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award.

*Chairman, Nominating Committee:*

R. V. BOUCHER

*Agricultural and Biological Chemistry  
Pennsylvania State University  
University Park, Pennsylvania*

## FOREWORD

### *Concerning Supplements to* The Journal of Nutrition

To meet the need for publication of meritorious but unusually long manuscripts the *Journal of Nutrition* instituted in April 1954 the policy of publishing such papers in the form of supplements to regular issues. The authors provide the full cost of such publication. For a more complete statement regarding this policy see volume 52, Supplement 1, April, 1954.

GEORGE R. COWGILL, *Editor*



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# TRYPTOPHAN-NIACIN RELATIONSHIPS IN MAN

STUDIES WITH DIETS DEFICIENT IN RIBOFLAVIN AND  
NIACIN, TOGETHER WITH OBSERVATIONS ON THE  
EXCRETION OF NITROGEN AND NIACIN METABOLITES

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# TRYPTOPHAN-NIACIN RELATIONSHIPS IN MAN

STUDIES WITH DIETS DEFICIENT IN RIBOFLAVIN AND NIACIN,  
TOGETHER WITH OBSERVATIONS ON THE EXCRETION  
OF NITROGEN AND NIACIN METABOLITES<sup>1</sup>

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(Received for publication December 13, 1955)

Although the prevalence of pellagra in areas where maize formed a considerable portion of the diet had been observed for several centuries, it is only in recent years that it has been possible to attempt to define the relative roles of niacin (Smith, '42), riboflavin (Sebrell and Butler, '38; Horwitt et al., '49b; Hills et al., '51) and tryptophan (Krehl et al., '46; Elvehjem, '48 and Goldsmith et al., '52) in the etiology of this disease. From these studies it would appear that the association of corn products with pellagra is more a function of the low tryptophan content of corn than of any "toxin" (Woolley, '46). The possibility remains that some links in this puzzle may still be missing.

<sup>1</sup>This project was supported by grants from the National Vitamin Foundation, Inc., and was sponsored by the Committee on Vitamin Studies at Elgin State Hospital, Food and Nutrition Board, National Research Council. During the period of this study, the committee was constituted as follows: Dr. Russell M. Wilder, chairman; Dr. O. A. Roney, Dr. E. S. G. Barrer, Dr. R. S. Goodhart, Dr. M. R. Harshbarger, Dr. A. C. Ivy, Dr. R. M. Kark, Mr. Paul Livingston, Dr. Fred Lott, Dr. A. A. Lieberman, Dr. D. L. Stokberg and Dr. Philip Whiteman.

<sup>2</sup>U. S. Public Health Service Fellow, 1952-53. Now at Mayo Clinic, Rochester, Minn. Dr. H. H. Hart was a U. S. Public Health Service Fellow on this project from June, 1953 to March, 1955, during which period he made important contributions.

This report will describe that part of Elgin National Research Council Project no. 3 which dealt with attempts to evaluate man's needs for niacin and tryptophan in diets low in corn products and both adequate and deficient in riboflavin. In order to decrease the level of tryptophan in the basal diets without using large amounts of corn meal, it was necessary to keep the protein intake at a low level. As a consequence of either this low protein level or of some other factor, changes in liver functions were observed after 5 months. These observations of liver dysfunction in man, produced and ameliorated by varying protein intake (Horwitt, '53; Horwitt, Rothwell and Kark, '53; Kark et al., '53) and the associated alterations in carbohydrate metabolism, will be discussed elsewhere, as these effects had no apparent direct relationship to niacin requirements although the implications of liver dysfunction did necessitate alterations in the original plan of the project. Though the liver dysfunction might possibly influence the rate at which the metabolites studied appeared in the urine in a short-term sample, such effects could not be noted in the 24- or 72-hour urine samples studied. Therefore, the data reported in this paper are considered to be similar to those which might be obtained from healthy subjects.

#### METHODS OF STUDY

As in previous long-term nutritional studies at Elgin (Horwitt et al., '48), the selection of subjects was made after evaluating the physical and mental qualities of a large number of mental patients. Choice was based upon chronicity of mental illness, adequacy of physical condition, and the presence of a reasonable amount of emotional stability. After a three-month trial period during which attitudes, cooperativeness and appetite habits were observed, 40 male subjects were divided into 5 experimental groups. The subjects were relatively young. All but one were 30 years or over, 8 were over 45 years and only three were over 60 years at the start of the study.

*Diets, dietary supplements and division of subjects*

The organization of the diet kitchen and the techniques used to control the dietary intake were similar to those previously described (Horwitt et al., '49c). The three basal diets<sup>2</sup> used are given in table 1. Three different sets of recipes were available so that, in effect, 9 different daily menus were rotated in an effort to provide some variety. A sample of the diet was homogenized and analyzed daily for nitrogen. Other constituents were determined at frequent intervals. The basal diets provided approximately 2300 Cal., 6.5 gm of protein nitrogen, 86 gm of fat, 5.8 mg of niacin, 265 mg of tryptophan and 415 µg of riboflavin.

As the quality of the protein in the diet may be of greater significance than the amount, it is pertinent to point out that the average amounts from wheat flour, zein<sup>4</sup> and gelatin used in the three basic diets were 15.1, 6.0 and 3.9 gm per day, respectively. It should also be noted that, since these diets contained approximately 41 gm of protein (6.59 gm of protein nitrogen) per day, they provided about 15 gm less protein than the diets of the previous Elgin studies. The 6.0 gm of zein and the 3.9 gm of gelatin were included as part of the total allotment in order to decrease further the tryptophan content of the diet. All of the above-mentioned data were calculated on the basis of 100% consumption of the diet. Actually, two subjects were allotted 90%, 21 subjects received 100%, three subjects received 110% and 5 subjects received 120% of all the ingredients of the basal diet in accordance with their appetite, size and personal preference as determined during the preliminary period. During the experimental period the percentage of the allotted food not consumed by any subject was negligible (less than 2%).

All subjects on the controlled diets received a daily vitamin supplement which contained 30 mg of ascorbic acid,

<sup>2</sup> For composition of diets see Horwitt et al., 1949c, 1950.

<sup>4</sup> Zein is a protein fraction of corn which is used as a source of protein in the diets. It is a mixture of several proteins and is not a single chemical entity. It is a mixture of several proteins and is not a single chemical entity.



TABLE 1  
*Constituents of daily diets used in Elgin project no. 3*

FOOD	SOURCE	WT. gm	DIET 1				DIET 2				DIET 3			
			Pro- tein	Tryp- tophan	Niacin	Ribo- flavin	Pro- tein	Tryp- tophan	Niacin	Ribo- flavin	Pro- tein	Tryp- tophan	Niacin	Ribo- flavin
Salt pork	Purchased	25	1.0	12	225	10	1.0	12	225	10	1.0	12	225	10
Farina <sup>1</sup>	Special	30	3.3	36	240	18								
Cornflakes <sup>1</sup>	Special	30					2.4	12	480	30	2.4	12	480	30
Pears	Home canned	100	0.2	2	100	20								
Pears	Home canned	50									0.1	1	50	10
Applesauce	Dried apples	100	0.4		200	20	0.4		200	20	0.4		200	20
Bread <sup>2</sup>	Special	120	8.6	70	1080	131	8.6	70	1080	131	8.6	70	1080	131
Margarine	A.P.	40	0.2				0.2				0.2			
Jelly	Homemade	60	0.1		120	12	0.1		120	12	0.1		120	12
Beef	Round <sup>3</sup>	25	4.9	69	1175	42	4.9	69	1175	42	4.9	69	1175	42
Potatoes	Varied	100	2.0	16	1200	40					2.0	16	1200	40
Spaghetti <sup>4</sup>	A.P.	50					6.4	51	1000	30				
Rice	Boiled	30	2.3	24	480	9	2.3	24	480	9	2.3	24	480	9
Cabbage	Special	67	2.6	24	209	12								
Sauerkraut	Varied	50					0.7	7	150	25				
Pudding	Homemade	50									0.6	6	50	30
Cream, 40%	Vanilla	100					0.1							
Sugar <sup>5</sup>	A.P.	25	0.6	5	25	28	0.6	5	25	28	0.6	5	25	28
Cake	Special	75												
Tomatoes	Home canned	50	0.5	5	350	15	0.5	5	350	15	3.2	26	266	15
Jello	34%	34	3.9				3.9							
Zein crackers	Special	55	9.9	33	270	15								
Zein rolls	Special	46					7.8	20	151	8				
Zein doughnuts	Special	106									9.6	38	341	19
Rhubarb	Home canned	100					0.4	4	100					
Dill pickles	Canned	25									0.2	2		15
Total (calculated) <sup>6</sup>			40.5	296	5674	372	40.3	290	5536	361	40.1	281	5692	411
Total (as analyzed)			40.6 <sup>7</sup>	274	5700	413	40.0	251	5570	422	41.1	268	5910	408
			±2.5	±20	±630	±37	±2.8	±8	±360	±35	±2.2	±17	±310	±34

<sup>1</sup> Prepared on special order by Quaker Oats Company.

<sup>2</sup> Baked from unenriched flour.

<sup>3</sup> Diets 1 and 3: round roast; diet 2: round ground.

<sup>4</sup> Unenriched, as purchased.

<sup>5</sup> Diets 1 and 2: 10 gm; diet 3: 10 gm.

<sup>6</sup> Agricultural Handbook no. 8.

<sup>7</sup> Grams nitrogen multiplied by 6.25.

0.6 mg of thiamine, 1.0 mg of pyridoxine, 3.0 mg of calcium pantothenate, 0.001 mg of vitamin B<sub>12</sub>, 0.1 mg of folic acid, 0.05 mg of biotin, 4000 I.U. of vitamin A and 400 I.U. of vitamin D.<sup>5</sup> In addition, tricalcium phosphate, 0.5 gm, and ferrous sulfate, 0.25 gm were given three times a week.

At the end of the three-month preliminary period, during which time base line data were accumulated, an attempt was made to distribute the 40 subjects uniformly with respect to age, weight, and psychological state, into the following 5 groups:

1. U group (unsupplemented). Eight subjects received the basal diet (one 90%, 6 100%, and one 120%) plus 0.1 mg of riboflavin per day; this small addition of riboflavin raised the intake of this vitamin to the level used in Elgin Project no. 2 (Horwitt et al., '49c). (After 38 weeks, in order to repair the ariboflavinosis which had developed, this group was supplemented with additional riboflavin, so that in effect, this group was thereafter equated with the "R" group.)

2. R group (riboflavin supplemented). Eight subjects received the basal diet (five 100%, two 110% and one 120%) plus 2 mg of riboflavin, daily.

3. NR group (niacinamide and riboflavin supplemented). Seven subjects received the basal diet (four 100% and three 120%) plus 2 mg of riboflavin and 10 mg of niacinamide, daily.

4. TR group (tryptophan and riboflavin supplemented). Eight subjects received the basal diet (one 90%, 6 100% and one 110%) plus 2 mg of riboflavin and 50 mg of L-tryptophan daily. The tryptophan supplement was doubled to 100 mg after 10 weeks.

5. HD group (Hospital diet). Nine subjects partook of the general hospital diet ad libitum, and were simultaneously subjected to the various procedures applied to the patients on the experimental diet. As a precaution they received a commercial vitamin mixture<sup>6</sup> three times a week which provided ap-

<sup>5</sup>The composition is property of H. C. Warr, La Brea, Inc., through the courtesy of Dr. J. L. Campbell, and Dr. M. D. Fildes.

<sup>6</sup>Abbott's Vita-Sol.

proximately 4 mg of niacinamide per day beyond that in the regular diet. The vitamin supplementation for this group was discontinued at the 38th week.

In former Elgin projects, it was relatively simple to maintain a given research plan similar to the one described above. In the present study only the first 37 weeks might be considered to have proceeded according to plan. The problem of liver dysfunction which developed after 5 months on the low-protein diet and the relative urgency of obtaining data which might be related to kwashiorkor made it expedient to alter the original goal somewhat in the hope that liver dysfunction might be proved and repaired without vitiating later interpretation of the data on niacin requirements. The following changes in the dietary supplements were made during the course of the experiment:

22nd to 38th week: An additional 0.5 mg of thiamine was given to half of the subjects in the U, R, NR and TR groups. Above average increases in blood lactic and pyruvic acids had been obtained after glucose ingestion and, although previous experience had indicated that the level of thiamine provided (approximately 0.9 mg per day) was not responsible for this metabolic change and that it was probably a manifestation of liver dysfunction, it was considered wise to supplement half the experimental subjects with thiamine for about 4 months in order to eliminate any doubt on this point. Further addition of 5 mg of thiamine, twice daily, was later tested on three subjects who had a marked disturbance of lactate and pyruvate metabolism (61st to 65th week) but increases in blood lactic and pyruvic acids were not reversed until after 30 gm of lactalbumin was given as a supplement, or a diet high in protein foods was fed.

39th to 61st weeks: lactalbumin, 10 gm daily, was administered to one-third of the subjects in the U, R, NR and TR groups, hereafter called subgroup I.

39th to 52nd weeks: L-lysine, 500 mg twice daily, was administered to another one-third of subjects, hereafter called

In order to assure this, schizophrenic men were selected for their ability to cooperate and they were housed and fed in a separate unit at the Hastings State Hospital. A series of rotating menus was developed so that the daily nutrient intake varied little though the diet was not particularly monotonous. An average level of 400 to 450 mg of cholesterol daily was aimed at and special cookies were provided which could add to this about 1000 mg daily of pure cholesterol when desired. The mode of life of the subjects was carefully standardized in regard to exercise, recreation, and so on, and this standard was maintained 24 hours a day by a special staff of aides.

After a month of standardization in the metabolic unit, subsisting on measured portions of the regular hospital diet, the men subsisted on the experimental diet for 8 weeks during which time all items of food were measured as served and each man's rejections or extra servings were also recorded. Careful attention was given to maintain the food consumption at the calorie balance point.

In experiment 54 A, 13 men consumed 374 mg of cholesterol daily for the first 4 weeks and then received an average of 1369 mg daily for the final 4 weeks. In experiment 54 B, 14 men went through the reverse order of change in cholesterol intake. The average values for dietary items of interest and for the serum total cholesterol at the end of each 4-week period are given for both experiments in table 8. There are suggestions of a trivial response of the serum cholesterol to the diet but in neither experiment is this statistically significant.

*Surveys in Sardinia.* On the Island of Sardinia the dietary pattern of the general population is very uniform in most respects and the consumption of cholesterol in the diet is relatively trivial except for that provided by eggs which are eaten in widely varying amounts. Hence, in dietary surveys it is readily possible to separate population samples into relatively low and high cholesterol intakes with the rest of their diets being substantially the same.

were discharged from unit in 69th, 69th and 38th week, respectively, for unrelated clinical reasons.

The above separations are not considered to be directly related to the experimental procedures but are more a function of normal turnover. The fact that several of the separations came from the "U" group is probably not significant, as their behavior was consistent with their pre-experimental records. There was some question about the accuracy of the food intake of subject R7-110. Consequently, data from this individual have been omitted from the final tabulations.

### *Tests employed*

Some of the procedures used were:

*Nitrogen.* A complete day's diet (100%) was acidified with acetic acid and homogenized. Samples of the homogenate were analyzed for nitrogen by a modification of the semi-micro method of Redemann ('39). Aliquots of the same homogenate were used for the other dietary assays listed below. The analyses of nitrogen in the urine were made on 72-hour collections. These three-day samples were acidified, cooled or frozen, depending upon when they were to be analyzed and used for all the urine analyses listed.

*Fat in the diet.* This was estimated by extraction with a 3 to 1 alcohol-ether mixture. The portion of the dried extract soluble in petroleum ether was considered "fat."

*Riboflavin.* The riboflavin in the diet and in an acidified aliquot of the 72-hour urine sample was analyzed by the microbiological technique of Roberts and Snell ('46). Riboflavin in the red blood cells and plasma was determined by the method of Burch et al. ('48) and will be discussed in a separate communication.

*Nicotinic acid.* The microbiological method used was similar to the U.S.P. modification of the method of Snell and Wright ('41). Because of the high fat content of the diet it was necessary first to extract most of the lipids with an alcohol-ether mixture.

## DISCUSSION

The foregoing evidence is definitive, we think, in showing that variations in the intake of cholesterol over the whole range of natural diets do not influence the serum level of physically normal adult men so long as other elements in the diet are constant. The results of experiments on 5 normal subjects reported by Mayer et al. ('54) are in agreement for the limited range of cholesterol intakes tried. The findings, too, of Kinsell et al. ('52) and of Ahrens et al. ('55) on hospital patients are confirmatory though the formula diets used in these experiments are radically different from any natural diets and are not, therefore, fully comparable.

According to the results of the carefully controlled experiments of Heymann and Rack ('43), the same rule applies to infants and children. In regard to women, Moses ('52) and Moses et al. ('52) found that the addition of 2 gm of cholesterol to the daily diet of pregnant women did not increase the normal trend to hypercholesterolemia in pregnancy.

The findings in two series of experiments reported in the literature may seem to be in disagreement but on closer scrutiny the results cannot be cited as showing an effect on dietary cholesterol. Okey and Stewart ('33) obtained a small positive response in young women when the yolks of two eggs were added to the daily diet but the fact that this involved a daily addition of about 100 Cal. of fats was not controlled. Similarly, in a prolonged experiment on 60 volunteers (Groen et al., '52), the dietary cholesterol changes were accompanied by substantial changes of the diet in other respects. For example, the "high" cholesterol diet (940 mg daily), provided 100 gm of proteins, 128 gm of fats and 2618 Cal., while for the "standard average" diet the subjects consumed an average of 75 gm of proteins, 99 gm of fats and 2391 Cal. Gillum et al. ('55) reporting on a survey on men and women aged 50 to over 80 years found that the dietary and serum cholesterol values were correlated with a coefficient value of  $r = +0.12$ . This value may be statistically significant because of the large number (53) of subjects,

*Bromsulfalein retention time.* The percentage of bromsulfalein remaining 45 minutes after injection of 5 mg per kilo was determined photometrically.<sup>7</sup>

*Eosinophils.* The extent of eosinopenia produced by adrenocorticotrophic hormone (ACTH) was determined by taking an eosinophil count on venous blood before and 4 hours following intramuscular injections of 25 mg of ACTH according to the method of Thorn et al. ('48). Results reported by Hiatt et al. ('52).

*Packed cell volume, albumin/globulin ratios, hemoglobin, erythrocyte counts, differential counts, gastric hydrochloric acid, serum cholesterol, N.P.N. and urinary sediments.* Determined quantitatively by standard techniques described in Hepler ('49).

Urine was analyzed qualitatively for pathological constituents, such as hemoglobin, albumin and glucose.

The *clinical tests* used included frequent examinations of the skin, oral cavity, cardiovascular system, metabolic rate, blood pressure, pulse rate, and gastrointestinal and liver functions. An attempt was made to evaluate the effects of ultra-violet and infra-red light exposure. The latter experiments were motivated by the findings of Smith and Ruffin ('37) that typical dermatitis could be produced in a pellagrin by exposure to heat as well as sunlight. The subjects were weighed before bedtime every Saturday and more often when a weight change was suspected.

#### OBSERVATIONS

No change in mental status was observed beyond the variations which might be anticipated in two years (preliminary plus experimental periods) in a group of patients with dementia praecox. There was no apparent diminution in appetite or change in bowel function (patients' complaints were practically non-existent). During the 35th week a subject in the "U" group (riboflavin- and niacin-deficient) with a rather

<sup>7</sup> These were read by Miss D. Rix in Dr. R. M. Kark's laboratory at the University of Illinois in order to facilitate comparison with other liver function tests being conducted simultaneously.

also appeared to be independent of the cholesterol intake in the diet. For example, in the Cagliari survey the mean percentage of the total serum cholesterol represented by that in the beta lipoprotein fraction, and its standard error, proved to be  $75.93 \pm 0.86$  and  $75.67 \pm 1.04$  in the low- and in the high-cholesterol-intake groups, respectively.

Finally, it may be asked why the human serum concentration of cholesterol is so remarkably independent of the amount of cholesterol supplied in the diet. After all, a daily intake of 1 gm of cholesterol, which characterizes some high-cholesterol diets, is something like a daily supply equal to 10% of the total amount of cholesterol in the entire blood volume. A very considerable proportion of this is absorbed from the intestine. But much more than this exogenous supply may be provided in the bile poured into the intestine and it would seem that this endogenous supply, synthesized by the liver, is easily regulated to adjust to the exogenous variations. A very different state of affairs prevails in the rabbit and the chick, of course, but it seems that most carnivores resemble man in this respect.

#### SUMMARY

1. Two cross sectional surveys in Minnesota on young men and 4 on older men showed no relationship between dietary cholesterol and the total serum cholesterol concentration over most of the ordinary intake range characteristic of American diets.

2. Two surveys on the Island of Sardinia failed to show any difference in the serum cholesterol concentrations of men of the same age, physical activity, relative body weight and general dietary pattern but differing markedly in cholesterol intake.

3. Careful study during 4 years of 33 men whose diets were consistently very low in cholesterol showed that their serum values did not differ from 35 men of the same age and economic status whose diets were very high in cholesterol.



degree of involvement noted before riboflavin (2 mg per day) was added to their regimen. Analyses of riboflavin in the urine which produced data which compared well with those previously reported (Horwitt et al., '49a) will be discussed in a subsequent publication. It is significant that although the present diet differed from that in the previous study in that vitamin B<sub>12</sub> was provided as a supplement and niacin was quite low, there were no noticeable differences in either the development or the repair of ariboflavinosis.

TABLE 2  
*Relative pathology of subjects on riboflavin depleted diet*

SUBJECT	ORAL CHANGES (Angular stomatitis, glossitis, mucosal erythema)	SCROTAL DERMATITIS	CONJUNCTIVITIS AND SEBORRHEA
U1 (100) <sup>1</sup>	+	+	0
U2 (100)	0	0	0
U3 (90)	+++	++	0
U4 (100)	±	0	0
U5 (100)	++++	++++	++++
U6 (100)	+++	+++	+++
U7 (120)	0	0	0
U8 (100)	+++	++	0

<sup>1</sup> Figures within parentheses indicate the percentage of food allotted.

A patient with the most severe scrotal dermatitis and penile involvement requires special comment. At the time supplementation was begun he showed unilateral angular stomatitis, diffuse erythema involving the tongue and buccal mucosa, retroaural seborrhea, and moderately severe scrotal dermatitis. During the first week after the daily supplementation with 2 mg of riboflavin there suddenly appeared severe blepharitis and conjunctivitis with photophobia, seborrhea and cracking of the skin in the nasolabial folds, marked bilateral angular stomatitis and cheilosis, and the severe penile and scrotal dermatitis described above. These lesions gradually cleared during the next 4 weeks leaving no apparent residue. Another patient with very marked scrotal dermatitis also had seborrhea and conjunctivitis, but to a less severe degree.

possible to carry out the work in Sardinia and in this operation we were greatly aided by Doctor Franco, Dr. Alfonso del Vecchio, and Dr. Henry L. Taylor and Mrs. Margaret Haney Keys. Most of the serum cholesterol measurements were made by Miss Erma V. O. Miller, Miss Laura Werner and Miss Nunzia Corrao. Mrs. Nedra Foster helped to plan the diets and analyze the food consumption records from Hastings, and helped analyze the blood serums. Mr. Norris Schulz aided in some of the statistical work.

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not reach minimum levels until after the experimental diet had been fed for more than three months. Thus, after two weeks on the experimental diet, the average excretion of nitrogen in the urine was 6.18 gm for the 100% group, which ate

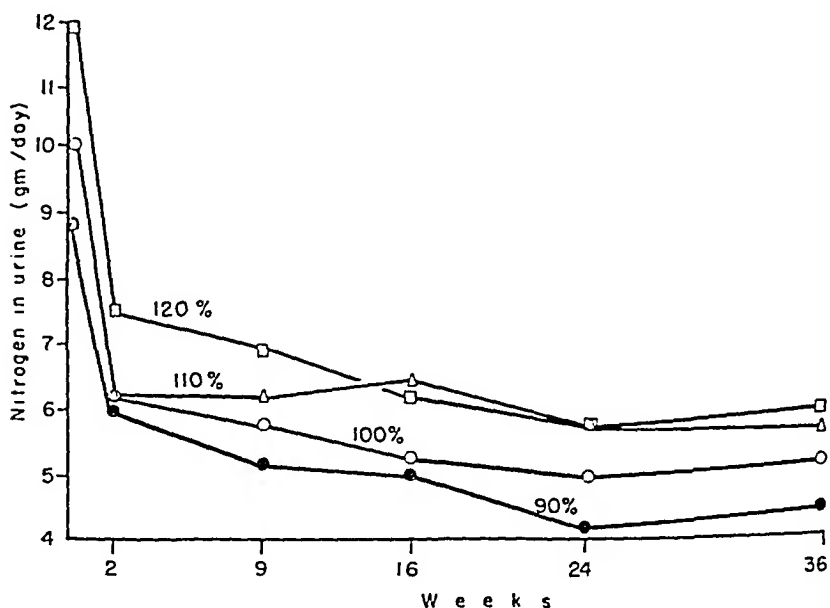


Fig. 1 Average urinary nitrogen excretions at 4 levels of nitrogen intake during first 36 weeks of controlled feeding; the 90, 100, 110 and 120% diets provided average of 5.85, 6.50, 7.15 and 7.80 gm nitrogen per day, respectively.

6.5 gm of nitrogen per day, and 7.54 gm for the 120% group which ate 7.8 gm of nitrogen per day. (The 110 and 90% groups gave parallel data but they represented only two subjects on each intake.) If one assumes that about 1 gm of nitrogen<sup>8</sup> was excreted in the feces, daily, then all these sub-

<sup>8</sup>Such an estimation of fecal nitrogen which is unacceptable in short-term nitrogen balance studies might be considered tolerable under the long-term plan of this experiment. The extra effort and expense that would have been involved in the collection of feces from 40 subjects would have made it impossible to conduct other phases of this project that appeared to show greater promise of producing significant data. Maximum variations in fecal nitrogen that might be expected in men repeating the same diet for many months do not appear to be large enough to have an important effect on the trends observed.

# PAIR FEEDING AS A CONTROL PROCEDURE IN METABOLIC STUDIES OF THE X-IRRADIATED RAT <sup>1</sup>

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FOUR FIGURES

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Whenever an experimental animal shows a simultaneous decrease in food intake and loss of body weight, one is confronted with the problem of discovering the extent to which inanition determines the final experimental result. This is a major problem in the study of the metabolic effects of total body x-irradiation in the rat.

A common way of circumventing this problem is to employ pair-feeding techniques which equate the food intakes of the control and experimental animals by using either: (a) the pair-starved control, i.e., reducing the food intake of the control group so as to parallel that of the experimental group, or (b) the tube-fed experimental and control, i.e., forcibly maintaining a constant food intake in the experimental as well as the control animals. There is little doubt that pair-feeding procedures will equate the amounts of food passing through the mouth of the control and experimental animals. A more critical problem is the extent to which these procedures equate the digestive and metabolic mechanisms within these different animals. It is to this problem, as it applies to the acutely x-irradiated rat, that this work is addressed.

<sup>1</sup> This work was supported under terms of Contract AT(11-1)-106 between the Atomic Energy Commission and the University of Minnesota.

TABLE 3

*Urinary nitrogen, in grams per day, excreted by those subjects on 100% dietary intake who were supplemented with 10 gm of lactalbumin before being given 30 gm of lactalbumin*

WEEKS	PRELIMINARY PERIOD			BASAL DIETS <sup>1</sup>				BASAL DIETS PLUS 10 GM LACTALBUMIN <sup>2</sup> STARTED AT 38TH WEEK		BASAL DIETS PLUS 30 GM LACTALBUMIN <sup>2</sup> STARTED AT 61ST WEEK	
	-12	-2	2	9	16	24	36	46	59	75	85
Subject	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
U 4	9.3	10.0	6.2	5.5	5.7	5.3	5.0	6.0	6.5	10.4	7.7
R 2	7.5	8.1	5.3	5.8	5.4	5.6	5.5	5.8	6.6	9.0	7.8
R 5	4.6	5.2	4.6	4.6	3.7	3.6	3.6	4.5	5.4	6.2	6.8
NR 3	8.8	8.3	5.7	5.2	4.9	4.8	4.2	4.6	5.1	7.8	8.3
TR 3	12.9	11.8	5.9	5.8	5.0	4.8	4.5	5.0	.. <sup>4</sup>		
TR 6	10.0	12.4	6.9	6.3	5.7	5.6	5.7	6.5	6.3	9.4	9.0
Average	8.9	9.3	5.8	5.5	5.1	5.0	4.8	5.4	6.0	8.6	7.9

<sup>1</sup> Basal diet provided an average of 6.5 gm of nitrogen.

<sup>2</sup> The 10 gm of lactalbumin provided 1.26 gm of nitrogen.

<sup>3</sup> The daily supplement of 30 gm of lactalbumin was difficult to ingest and these data are probably low.

<sup>4</sup> Subject TR 3 transferred to hospital diet in 52nd week.

the body weights observed when both the control and irradiated animals are fed a constant amount of milk diet administered twice daily by stomach tube. When the food intake was maintained constant by this means, it was again found that the weight loss of the irradiated (group 3) and control (group 4) animals are, within experimental limits, equal. Smith, Ackermann and Smith ('52) have reported the same effect when a diet containing a pancreatic digest of

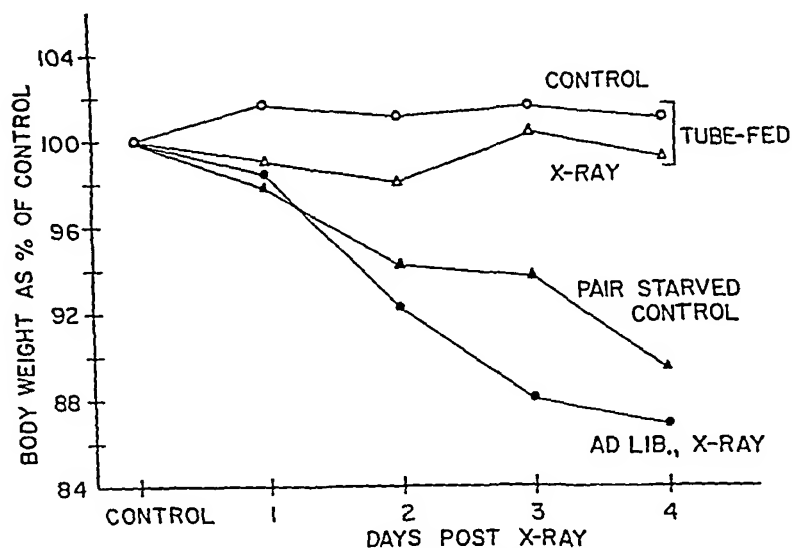


Fig. 1 Total body weight changes observed in x-irradiated and pair-fed rats.

casein<sup>4</sup> was tube-fed. Figure 1, then, summarizes evidence which can be given as proof that the weight loss of radiation sickness is due quantitatively to starvation.

From the evidence in figure 1, it would seem possible to avoid all weight loss problems by tube-feeding the animals. When the distribution of tissue weight is studied by dissection procedures, quite a different picture emerges. This is largely due to the marked difference in the weight of the gastrointestinal contents and the difference in the loss of muscle mass

<sup>4</sup> Amigen, Mead, Johnson and Co., Evansville, Indiana.

TABLE 4

*Urinary excretion of N<sup>5</sup>-methylnicotinamide (mg/day)*

(U, R, NR, TR and HD refer to original classification of subject as being in the unsupplemented, riboflavin, niacin plus riboflavin, tryptophan plus riboflavin or hospital diet groups, respectively. Data are subgrouped according to percentage of food allotted and similarity of supplementation in 46th week.)

SUBJECT		PRELIMINARY PERIOD		TIME IN WEEKS									
No.	Body wt.			2	9	16	24	36	46 <sup>1</sup>	59	75	84	95 <sup>2</sup>
	kg	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day
U2-100	61	4.04	2.49	1.53	2.00	1.37	1.07	0.69 <sup>2</sup>	(0.49) <sup>4</sup>	4.76 <sup>3</sup>	5.57	8.01	8.01
U5-100	53	3.76	4.26	0.96	1.20	0.85	1.00	0.64 <sup>2</sup>	1.05 <sup>4</sup>	0.66 <sup>3</sup>	0.69	4.28	4.28
U6-100	55	3.66	1.69	1.19	1.29	1.42	0.56	0.49 <sup>1</sup>	0.96 <sup>3</sup>	1.12 <sup>6</sup>	0.91	3.72	3.72
U-8-100	59	6.30	5.90	1.56	1.53	0.77	0.86	0.98 <sup>2</sup>	1.29 <sup>4</sup>	...	...	...	...
U3-90	63	5.39	1.63	2.06	1.10	1.96	1.28	6.06 <sup>10</sup>	3.25 <sup>11</sup>	6.24 <sup>12</sup>	6.50	9.14	9.14
U1-100	69	7.63	2.47	2.37	2.10	1.42	1.49	1.81 <sup>13</sup>	2.61	3.14 <sup>14</sup>	3.33	7.03	7.03
U4-100	63	3.42	2.23	1.16	1.10	1.25	0.60	2.04 <sup>12</sup>	2.33	5.01 <sup>5</sup>	2.96	3.00	3.00
U7-120	65	5.49	3.61	1.68	2.03	2.67	1.63	1.61 <sup>12</sup>	2.13	3.87 <sup>15</sup>	4.79	4.84	4.84
R1-100	61	5.41	2.54	1.18	1.33	0.96	0.47	0.38 <sup>7</sup>	0.63 <sup>8</sup>	...	...	...	...
R3-100	59	3.15	1.84	1.28	1.39	1.11	1.40	0.87 <sup>2</sup>	1.23 <sup>4</sup>	1.38 <sup>6</sup>	1.34	8.43	8.43
R4-100	58	3.44	2.00	0.59	1.07	0.88	0.87	0.82 <sup>7</sup>	0.59 <sup>8</sup>	0.98 <sup>6</sup>	0.81	3.86	3.86
R6-120	71	5.69	3.22	2.54	1.75	1.76	1.17	0.77 <sup>2</sup>	0.75 <sup>4</sup>	1.87 <sup>11</sup>	2.11	3.08	3.08
R2-100	64	2.58	1.55	0.72	0.61	(0.26) <sup>16</sup>	(1.04) <sup>16</sup>	0.61 <sup>12</sup>	0.72	3.00 <sup>11</sup>	1.93	8.15	8.15
R5-100	65	...	2.59	1.04	0.96	0.80	0.92	(0.55) <sup>12</sup>	1.09	1.47 <sup>11</sup>	1.91	2.40	2.40
R8-110	55	5.16	2.37	1.60	1.21	0.75	1.05	1.92 <sup>12</sup>	1.42	1.45 <sup>6</sup>	1.20	5.18	5.18
NR1-100	73	4.69	6.37	5.39	5.36	5.27	6.12	3.22 <sup>7</sup>	2.79 <sup>8</sup>	5.47 <sup>11</sup>	5.53	6.02	6.02
NR2-100	62	4.87	6.91	4.99	6.90	4.25	4.00	4.03 <sup>2</sup>	4.27 <sup>11</sup>	9.65	7.03	8.45	8.45
NR5-100	61	3.87	3.36	4.11	3.22	2.81	3.38	2.08 <sup>7</sup>	2.55 <sup>8</sup>	2.36 <sup>6</sup>	3.57	5.93	5.93
NR4-120	69	5.05	6.78	5.47	5.15	3.32	4.04	4.72 <sup>2</sup>	5.47 <sup>4</sup>	6.98 <sup>15</sup>	5.98	5.62	5.62
NR7-120	77	...	4.78	3.08	4.83	4.76	3.16	3.57 <sup>2</sup>	6.23 <sup>4</sup>	5.76 <sup>14</sup>	4.44	5.06	5.06
NR3-100	58	3.44	6.24	6.56	5.09	4.55	3.52	5.43 <sup>12</sup>	5.34	7.23 <sup>11</sup>	6.47	8.48	8.48
NR6-120	63	4.53	3.88	4.78	5.10	2.95	5.44	6.30 <sup>12</sup>	3.39	5.53 <sup>11</sup>	5.30	6.38	6.38

misleading information concerning body weight loss and metabolic balance.

*Pair-starved control.* The weight loss of the irradiated rat and its pair-starved control were found to be roughly parallel as shown in figure 1. Here again the parallelism disappears when the animals are dissected and the tissue weight changes compared (fig. 2).

Again, one of the major differences comes in the weight of material retained in the stomach. Much of the weight loss of the starving rat is due to that of gastrointestinal contents.

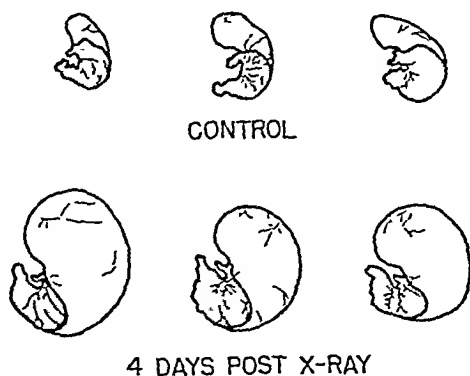


Fig. 3 General size and appearance of the stomachs of tube-fed rats at 18 hours after their last feeding. The cross-hatched area represents the pyloric portion.

In many rats this loss actually exceeded that of the loss in body weight during the first 24 hours; indicating that the total tissue weight actually increased slightly in the initial period. This slight increase may be associated with a change in hydration, since the water intake increased by two- to three-fold during the first day of starvation.

Figure 4 shows the basic differences in the weight changes observed in these two conditions. Several facts stand out. The main weight loss following irradiation is due to a loss of muscle mass. In the starving animal, however, there is no loss of muscle until after the third day, while the main loss of weight results from a decrease in gastrointestinal



mented with 10 mg of niacinamide) increased to 5.39 mg per day. At the same time, the HD group which received the hospital diet plus a supplement of 10 mg of niacinamide, three times a week, reached a plateau level of 6.44 mg.

The R-100 group was close to its minimum average excretion level at the 9th week with only minor fluctuations thereafter. The U-100 group average took somewhat longer to come

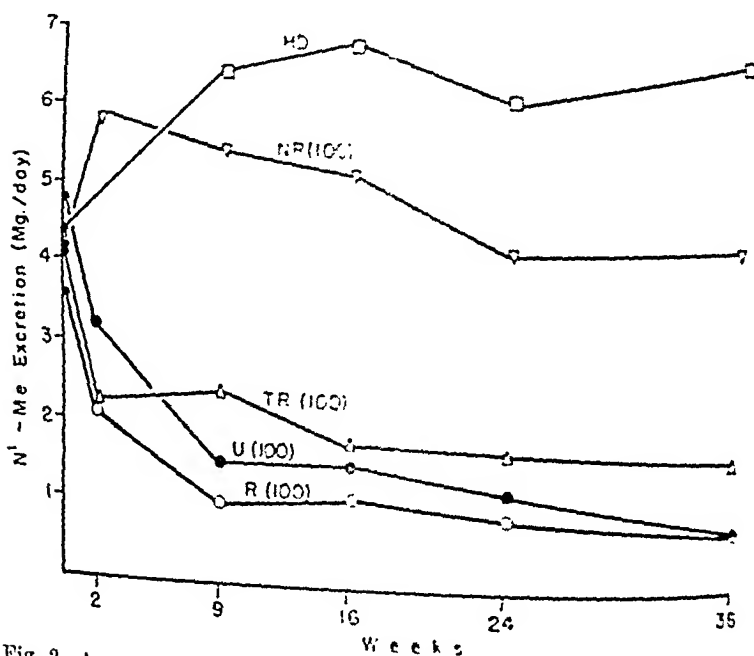


Fig. 2 Average daily excretion of N¹-methylnicotinamide of the experimental groups.

down to a similar low level of about 1.0 mg N¹-Me per day but this time difference is probably related to the higher initial reserve shown by the U-100 subjects in the preliminary period. At two weeks the average values for the TR-100 (2.30 mg), U-100 (3.17 mg) and R-100 (2.12 mg) groups were still a function of the pre-experimental levels. The data from the 9th week gave definite confirmation of the conversion of tryptophan to N¹-Me even though the L-tryptophan supplement was only 50 mg per day at that time. L-Tryptophan supplement

procedures allow one to eliminate the effects of inanition from the other effects of radiation, and thus simplify the interpretation of the data. The difficulties of this approach can best be illustrated by taking a specific example. In electrolyte balance studies (Bowers et al., '53), the irradiated rat has been compared with his pair-starved control. To be exact, the pair-starved control balances were subtracted from the irradiated animal balances, and the data expressed as balance differences. As might be expected from figure 4, during the first stress days the irradiated animal loses substantial amounts of muscle potassium, and the starved animal loses substantial amounts of gastrointestinal potassium. In this comparison, then, one subtracts a muscle potassium loss from a gastric potassium loss, and labels the difference as the potassium loss attributable to "pure" irradiation effects. Quite obviously this use of the pair-fed control can only lead to gross confusion concerning the "pure" metabolic effects of irradiation. Furthermore, the lack of appropriate control data prevents a reinterpretation of the experimental data on any other basis.

All of this raises some very practical problems concerning the conditions under which it may be justifiable to use this pair-feeding technique. Physiological and statistical considerations suggest that the following three conditions should be met: (1) The net effects (total-body potassium losses) of the stress (irradiation) are similar to those observed in starvation, (2) these outward effects are caused by the same metabolic alterations in each case, and (3) there is evidence of the independence of (lack of interaction between) the stress (irradiation) and starvation effects.

Since the starving and irradiated animals are losing equivalent amounts of potassium (Bowers et al., '53), the first condition is satisfied. However, the clear difference in the nature of the internal mechanisms involved points to a failure to meet the second condition. This is not an isolated or unique case. In a number of the situations which have received careful study, the metabolic changes in the irradiated animal were

in an unsupplemented group (U or R group) who received 120% of the basal diet was being allotted 318 mg of tryptophan, which is not much less than 338 mg of tryptophan which the subject on 90% intake was receiving in the TR group (basal diet plus 100 mg of tryptophan).

Table 4 shows that several of the subjects had low excretions of N<sup>1</sup>-Me which approached the upper limits of those reported by Goldsmith et al. ('52), levels which were associated with clinical pellagra on a corn diet. In the latter study, excretions of 0.5 and 0.6 mg of N<sup>1</sup>-Me were obtained in 50 to 60 days. In the present study, only one subject (R2-100) excreted less than 0.96 mg at 16 weeks and although, between 46 and 59 weeks, 6 subjects (U2-100, U5-100, U6-100, R1-100, R2-100 and R5-100) recorded excretions of 0.6 mg or less, clinical evidence of pellagra was not apparent. These results are not inconsistent with similar observations on wheat diets which were being conducted at Tulane University (Goldsmith et al., '52) at about the same time. In the latter study, the depletion period was 95 days at which time some of the subjects in the Elgin study had also reached their minimal levels of N<sup>1</sup>-Me excretion.

*Effects of lysine supplementation on N<sup>1</sup>-methyl-nicotinamide excretion*

The apparent changes in N<sup>1</sup>-Me excretion observed (table 4, footnote 10) when 1 gm of L-lysine per day was given (39th week) to subjects U2-100, U5-100, R3-100 and R6-120 deserve special comment. These subjects had a decrease in N<sup>1</sup>-Me excretion, presumably due to the amino acid imbalance associated with lysine supplementation (Koeppe and Henderson, '55). These suggestions of decreases were in contrast to larger excretions obtained at the same time, in 6 subjects in the same group who were supplemented with 10 gm of lactalbumin per day, although two of these subjects (R2-100 and R5-100) needed more time to show the ameliorative effect of the lactalbumin. The two subjects in the TR group (TR4-100 and TR7-100) who received 1 gm of L-lysine per day

- GOODMAN, R. D., A. E. LEWIS AND E. A. SCHUCK 1952 Effects of x-irradiation on gastrointestinal transit and absorption availability. *Am. J. Physiol.*, 169: 242-247.
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the 10 gm of lactalbumin used <sup>10</sup> was approximately 200 mg. As the amount of tryptophan in 10 gm of lactalbumin is about twice the 100 mg added daily to the basal diet given to the subjects in the TR group, the increased efficiency of the lactalbumin supplementation on N<sup>1</sup>-Me can be calculated as a function of the additional tryptophan so provided. To estimate the comparative value of tryptophan from lactalbumin and of the 10 mg of niacin given to the NR subjects, one can compare the pre-lactalbumin supplementation data from the 36th week with data obtained during the 59th week. The average increase in the excretion of N<sup>1</sup>-Me due to the addition of 10 gm of lactalbumin in the 39th week to 6 subjects who appear to have given reliable data (U1-100, U4-100, U7-120, R8-110, NR3-100 and TR6-100) was approximately 1 mg per day. The difference between the amounts excreted by the NR-100 and U and R-100 groups was approximately 3 mg per day (see fig. 2). On this basis one might regard 1 mg of niacin as being equivalent to about 3 gm of lactalbumin or to about 60 mg of tryptophan provided in the form of lactalbumin. While this calculated result cannot be proved statistically significant from the data presented, it can serve as a reference point for estimating the niacin equivalence of tryptophan-containing compounds; some additional justification for this relationship is presented below in the section in which N<sup>1</sup>-Me excretion produced by ingestion of niacin or tryptophan is discussed. When lactalbumin replaced zein in the basal diet (52nd to 61st week, table 4, footnote 12), subjects U5-100, U8-100 and R3-100 showed increases in N<sup>1</sup>-Me excretion of 0.64 to 1.05, 0.98 to 1.29 and 0.87 to 1.23 mg, respectively, after 7 weeks. Since this tryptophan addition of approximately 140 mg per day followed the cessation of lysine supplementation, these data are difficult to evaluate.

#### *Urinary excretion of quinolinic acid, niacin and tryptophan*

The excretion data for quinolinic acid indicate that this analysis, though of theoretical importance, does not produce

<sup>10</sup> Labco Brand, purchased from The Borden Company (nitrogen content 12.6%).

# THE EFFECT OF PROTEIN LEVEL ON THE TRYPTOPHAN REQUIREMENT OF THE GROWING CHICK<sup>1</sup>

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TWO FIGURES

(Received for publication December 13, 1955)

Grau ('48) and Grau and Kamei ('50) demonstrated an increase, at a decreasing rate, of the absolute lysine and sulfur amino acid requirements of the growing chick when the protein level of the diet was increased. Almquist ('49) found that at supernormal protein levels the methionine requirement was a constant percentage of the protein; similar observations were made by Almquist and Merritt ('50) with arginine.

If all essential amino acids, even at supernormal levels, had to be supplied as a constant percentage of the protein, higher levels of proteins deficient in one or more amino acids would only serve to accentuate an imbalance. If there were, however, a decrease in the requirements, expressed as percentage of the protein, with increasing protein levels, it might be possible to overcome moderate amino acid deficiencies by feeding more of the deficient protein. Barnes et al. ('45) have shown with rats that by increasing the level of properly heated soy-flour, deficient in methionine, or wheat gluten, deficient in lysine, above what is generally considered optimum, a level of growth

<sup>1</sup> The experimental data in this paper are taken from a thesis submitted by the senior author to the Graduate School of the University of Illinois in partial fulfillment of the requirements of the Ph.D. degree in Animal Nutrition.

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Urinary excretion of quinolinic acid (mg/day)

SUBJECT No.	Body wt. kg	PRELIMINARY PERIOD	TIME IN WEEKS									
			2	9	16	24	36	46 <sup>1</sup>	59	75	84	95 <sup>2</sup>
		mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day
U2-100	61	3.88	4.56	4.33	4.46	2.92	3.60	4.51 <sup>3</sup>	3.29 <sup>4</sup>	3.68 <sup>5</sup>	3.78	3.55
U5-100	53	2.73	4.35	2.35	3.51	1.97	2.45	2.43 <sup>3</sup>	2.50 <sup>4</sup>	2.53 <sup>5</sup>	1.37	3.68
U6-100	55	3.19	3.31	2.23	3.60	3.56	3.01	2.67 <sup>3</sup>	2.62 <sup>4</sup>	2.87 <sup>5</sup>	1.80	2.38
U8-100	59	6.63	4.87	3.42	5.05	3.45	3.57	3.48 <sup>3</sup>	3.05 <sup>4</sup>	...	...	...
U3-90	63	3.77	3.66	1.94	4.04	1.87	2.26	...	...	2.55 <sup>12</sup>	1.68	2.94
U1-100	69	3.87	4.14	2.98	4.53	2.26	3.09	3.72 <sup>12</sup>	4.42	2.10 <sup>14</sup>	3.73	3.39
U4-100	63	3.33	3.63	2.50	4.43	1.96	2.63	3.60 <sup>12</sup>	3.10	3.17 <sup>14</sup>	2.58	3.16
U7-120	65	5.02	5.00	3.99	4.59	4.42	4.47	5.05 <sup>12</sup>	3.84	3.69 <sup>14</sup>	4.14	4.12
R1-100	61	3.29	3.45	1.87	3.42	2.18	3.25	2.51 <sup>7</sup>	2.03 <sup>3</sup>	...	...	7.73
R3-100	59	4.19	4.13	4.19	6.29	3.60	4.26	3.43 <sup>3</sup>	3.69 <sup>4</sup>	3.36 <sup>6</sup>	3.00	2.45
R4-100	58	3.15	3.76	3.00	4.12	2.86	3.86	3.48 <sup>7</sup>	1.74 <sup>3</sup>	2.97 <sup>6</sup>	2.48	2.96
R6-120	71	3.55	4.25	4.22	7.12	4.06	3.62	3.86 <sup>3</sup>	2.81 <sup>4</sup>	3.87 <sup>14</sup>	3.62	2.02
R2-100	64	3.07	3.55	3.21	4.66	3.41	4.74	3.54 <sup>12</sup>	2.59	3.11 <sup>14</sup>	3.28	3.25
R5-100	65	1.84	3.16	1.89	3.65	2.63	2.47	2.56 <sup>12</sup>	2.27	3.01 <sup>14</sup>	2.69	1.92
R8-110	55	4.91	4.62	3.42	4.75	2.90	4.34	3.91 <sup>12</sup>	4.16	4.12 <sup>6</sup>	4.06	4.33
NR1-100	73	3.82	4.38	3.30	4.28	2.61	3.59	3.28 <sup>7</sup>	1.12 <sup>3</sup>	4.09 <sup>14</sup>	3.41	3.91
NR2-100	62	6.30	8.61	7.88	7.53	8.61	6.55	6.46 <sup>3</sup>	7.57 <sup>11</sup>	6.72	7.32	6.81
NR5-100	61	3.00	3.92	2.79	4.18	3.91	3.83	3.44 <sup>7</sup>	2.84 <sup>3</sup>	2.51 <sup>6</sup>	2.84	3.98
NR4-120	69	4.70	4.62	3.61	5.12	6.01	4.42	4.08 <sup>3</sup>	4.11 <sup>4</sup>	4.33 <sup>12</sup>	4.88	4.71
NR7-120	77	2.49	4.20	3.26	4.97	4.60	3.53	3.76 <sup>3</sup>	3.05 <sup>4</sup>	2.75 <sup>14</sup>	3.84	5.93
NR3-100	58	4.63	6.01	5.15	5.67	3.83	5.10	5.35 <sup>12</sup>	3.77	4.61 <sup>14</sup>	5.12	6.02
NR6-120	63	4.32	5.41	3.84	5.04	4.52	4.69	5.51 <sup>12</sup>	4.22	3.49 <sup>14</sup>	4.77	3.88
TR1-100	58	3.68	5.19	4.96	3.42	4.89	3.48	4.12 <sup>7</sup>	2.62 <sup>3</sup>	1.26 <sup>14</sup>	3.66	4.02
TR4-100	61	5.53	5.88	4.64	3.91	5.54	5.06	5.23 <sup>3</sup>	3.71 <sup>4</sup>	4.84 <sup>14</sup>	4.99	4.41
TR5-100	76	4.69	4.40	3.95	4.22	3.13	4.51	4.22 <sup>7</sup>	3.26 <sup>3</sup>	3.68 <sup>14</sup>	8.81	4.44
TR7-100	76	5.28	4.43	5.36	3.95	4.60	4.70	3.76 <sup>3</sup>	3.65 <sup>4</sup>	3.93 <sup>14</sup>	4.89	5.58
TR8-90	57	4.02	4.60	3.73	3.91	3.73	3.71	3.70 <sup>7</sup>	2.39 <sup>3</sup>	3.97 <sup>14</sup>	3.75	3.91
TR2-110	68	3.90	4.86	5.09	4.87	3.36	3.43	...	...	...	...	...
TR3-100	65	3.92	3.79	3.02	3.34	3.79	4.19	3.65 <sup>12</sup>	3.08 <sup>11</sup>	2.62	3.57	4.15
TR6-100	62	4.45	4.03	3.63	3.57	4.20	3.35	4.64 <sup>12</sup>	3.65	3.75 <sup>14</sup>	2.84	4.15
HD1	70	4.18	...	...	...	...	...	3.87 <sup>12</sup>	4.48	4.98	5.46	4.09
HD2	54	3.68	...	3.95	3.46	4.83	4.41	4.17 <sup>12</sup>	3.02	4.87	3.52	3.57
HD3	63	3.67	...	3.91	3.43	3.83	3.00	3.71 <sup>12</sup>	6.02	3.85	2.86	4.23
HD4	70	...	...	...	...	...	...	5.47 <sup>12</sup>	5.42	7.47	5.12	8.57
HD5	65	3.55	...	4.58	4.40	4.34	3.72	4.39 <sup>12</sup>	2.85	4.62	3.86	3.97
HD6	57	2.88	...	3.99	4.67	4.10	3.73	3.55 <sup>12</sup>	3.21	3.27	3.50	3.66
HD7	62	4.22	...	...	...	...	...	1.87 <sup>12</sup>	3.28	2.69	...	...
HD8	53	3.74	...	...	...	...	...	3.80 <sup>12</sup>	3.32	7.99	8.50	12.06
HD9	72	4.75	...	...	...	...	...	10.34 <sup>12</sup>	4.97	4.43	3.39	7.57

See table 4 for description of footnotes.

cured sample used in these experiments was analyzed by the Kjeldahl-Wilfarth-Gunning method for nitrogen and microbiologically for tryptophan, using barium hydroxide hydrolysis and *S. faecalis*, according to a modification of the method of Miller and Ruttinger ('50). The alfalfa sample was found to contain 20% of protein ( $N \times 6.25$ ) and 0.30% of trypto-

TABLE 1  
Basal diets used in experiments 1 and 2

INGREDIENTS	DIET O	DIET A	DIET B	DIET C	DIET D
	%	%	%	%	%
Cerelose	57.92				
Dextrin		76.46	63.84	51.16	38.46
Casein, crude	18.00				
Casein, acid hydrolyzed <sup>1</sup>	2.00	10.90	22.42	34.00	45.60
Gelatin	12.24				
Alfalfa meal, sun-cured	3.00	3.00	3.00	3.00	3.00
Salt mixture <sup>2</sup>	5.34	5.34	5.34	5.34	5.34
Corn oil, refined	1.00	3.00	3.00	3.00	3.00
Choline Cl	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.30	0.10	0.20	0.30	0.40
L-Arginine HCl		0.50	1.00	1.50	2.00
Glycine		0.50	1.00	1.50	2.00
Total <sup>3</sup>	100.00	100.00	100.00	100.00	100.00

<sup>1</sup> HY-CASE, a salt-free product of Sheffield Chemical Company, Inc., Norwich, N. Y.

<sup>2</sup> Fisher et al. ('54a).

<sup>3</sup> Plus the following vitamins (milligrams per kilogram diet): thiamine HCl 100.0; riboflavin 16.0; niacin 100.0; calcium pantothenate 20.0; pyridoxine 6.0; folic acid 4.0; biotin 0.6; vitamin B<sub>12</sub> 0.02; inositol 100.0; para-aminobenzoic acid 2.0; ascorbic acid 250.0; Menadione 5.0;  $\alpha$ -tocopherol acetate 20.0; 10,000 I.U. vitamin A and 600 I.U. vitamin D<sub>3</sub>. Procaine penicillin G was added at the level of 15 mg/kg.

phan. The hydrolyzed casein was supplemented with methionine, arginine and glycine so that except for the small amount of protein supplied by the alfalfa meal, the ratio of all amino acids except tryptophan to each other did not differ materially in the 4 experimental diets. Due to the inefficient conversion of orally administered D-tryptophan to the L isomer, reported by Morrison ('55), only the natural isomer was used to supple-



TABLE 7

## Urinary excretion of L-tryptophan

SUBJECT No.	Body wt. kg	PRELIMINARY PERIOD	TIME IN WEEKS									
			2	9	16	24	36	46 <sup>1</sup>	59	75	84	95 <sup>2</sup>
		mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day
U2-100	61	9.6	8.9	11.9	10.8	9.1	9.1	10.6 <sup>3</sup>	6.0 <sup>4</sup>	26.7 <sup>5</sup>	16.0	12.6
U5-100	53	7.0	6.4	11.6	7.8	6.7	8.4	6.8 <sup>3</sup>	4 <sup>4</sup>	9.4 <sup>5</sup>	10.5	11.7
U6-100	55	10.0	12.0	13.2	12.6	11.7	12.3	12.0 <sup>7</sup>	7.4 <sup>8</sup>	15.7 <sup>6</sup>	14.3	17.2
U8-100	59	7.4	14.2	10.0	8.4	9.4	8.7	8.0 <sup>3</sup>	4.8 <sup>4</sup>	...	...	...
U3-90	63	10.3	10.3	11.7	10.2	10.3	10.4	12.6 <sup>10</sup>	...	23.6 <sup>12</sup>	17.8	16.4
U1-100	69	23.9	18.8	14.1	13.0	12.3	14.5	16.8 <sup>13</sup>	18.5	20.0 <sup>14</sup>	12.4	15.9
U4-100	63	11.9	14.7	16.6	14.2	13.7	13.0	16.4 <sup>13</sup>	17.7	18.6 <sup>5</sup>	11.5	13.3
U7-120	65	13.2	16.6	15.6	13.0	14.9	14.8	17.6 <sup>13</sup>	17.6	17.5 <sup>13</sup>	23.9	21.1
R1-100	61	12.1	18.7	12.9	11.4	12.4	13.4	12.0 <sup>7</sup>	20.0 <sup>8</sup>	...	...	26.0
R3-100	59	9.2	13.0	11.1	8.0	14.4	8.1	7.4 <sup>3</sup>	6.7 <sup>4</sup>	5.9 <sup>5</sup>	8.3	15.5
R4-100	58	12.6	19.5	16.3	13.4	13.2	16.2	13.5 <sup>7</sup>	8.9 <sup>8</sup>	7.2 <sup>6</sup>	14.7	25.5
R6-120	71	7.5	10.8	12.4	9.0	8.3	7.9	8.0 <sup>3</sup>	6.8 <sup>4</sup>	18.3 <sup>14</sup>	10.0	8.6
R2-100	64	6.7	6.7	11.3	8.8	9.4	8.3	8.2 <sup>13</sup>	...	9.5 <sup>14</sup>	14.7	12.9
R5-100	65	5.9	12.3	10.8	6.8	8.3	7.4	9.3 <sup>13</sup>	5.6	8.8 <sup>14</sup>	9.2	14.0
R8-110	55	7.4	8.7	11.8	9.5	8.4	6.1	8.8 <sup>13</sup>	10.2	11.6 <sup>6</sup>	11.9	14.2
NR1-100	73	19.3	22.7	18.8	17.5	16.7	16.8	14.9 <sup>7</sup>	9.9 <sup>8</sup>	8.1 <sup>14</sup>	19.8	25.2
NR2-100	62	8.0	7.8	8.4	6.0	6.9	5.6	4.9 <sup>3</sup>	6.8 <sup>11</sup>	12.9	9.3	27.5
NR5-100	61	15.8	9.9	17.0	10.1	11.6	9.1	9.2 <sup>7</sup>	18.0 <sup>8</sup>	11.2 <sup>6</sup>	9.0	15.9
NR4-120	69	9.3	16.5	14.3	12.7	11.4	11.2	11.3 <sup>3</sup>	5.3 <sup>4</sup>	19.4 <sup>13</sup>	14.2	17.3
NR7-120	77	14.9	13.7	20.8	19.4	24.0	19.2	19.8 <sup>3</sup>	18.4 <sup>4</sup>	21.9 <sup>14</sup>	18.5	24.3
NR3-100	58	15.2	11.7	10.8	8.7	9.7	8.4	7.8 <sup>13</sup>	12.2	11.9 <sup>14</sup>	10.4	13.2
NR6-120	63	12.1	16.6	13.2	10.8	12.8	12.1	15.1 <sup>13</sup>	18.0	12.6 <sup>14</sup>	12.0	18.4
TR1-100	58	7.8	11.8	13.7	7.8	7.7	7.3	7.1 <sup>7</sup>	7.2 <sup>8</sup>	9.9 <sup>14</sup>	7.5	15.2
TR4-100	61	11.8	19.0	17.3	14.4	24.6	17.8	14.8 <sup>3</sup>	6.1 <sup>4</sup>	16.2 <sup>14</sup>	20.0	31.0
TR5-100	76	19.2	20.9	20.2	18.6	23.8	22.2	22.2 <sup>7</sup>	19.4 <sup>8</sup>	16.6 <sup>11</sup>	20.0	28.6
TR7-100	76	12.4	16.3	19.4	14.9	12.9	14.5	11.8 <sup>3</sup>	11.3 <sup>4</sup>	19.4 <sup>14</sup>	15.9	20.4
TR8-90	57	7.6	7.8	14.0	7.5	7.6	7.8	8.9 <sup>7</sup>	8.5 <sup>8</sup>	10.3 <sup>14</sup>	8.2	11.1
TR2-110	68	10.1	9.8	17.0	12.2	10.3	12.8	...	...	...	...	...
TR3-100	65	12.2	13.4	11.4	9.1	12.3	12.0	11.4 <sup>13</sup>	13.3 <sup>11</sup>	12.5	11.6	14.3
TR6-100	62	7.9	15.1	12.0	8.7	8.3	8.2	9.4 <sup>13</sup>	5.0	10.7 <sup>14</sup>	12.1	13.8
HD1	70	9.8	...	...	...	...	18.6	17.1 <sup>13</sup>	16.9	18.5	18.6	16.2
HD2	54	8.2	...	30.6	11.6	14.5	20.5	14.5 <sup>13</sup>	...	20.2	12.3	17.8
HD3	63	11.7	...	31.6	35.6	20.2	...	23.6 <sup>13</sup>	...	20.6	18.2	26.7
HD4	70	9.2	...	...	...	...	...	11.4 <sup>13</sup>	...	13.6	13.0	14.0
HD5	65	7.1	...	16.6	11.7	9.7	9.8	10.0 <sup>13</sup>	...	10.7	12.2	15.0
HD6	57	10.9	...	18.5	15.0	13.2	13.6	15.6 <sup>13</sup>	...	22.8	20.0	18.1
HD7	62	7.7	...	...	...	...	...	21.3 <sup>13</sup>	...	17.3	...	...
HD8	53	7.2	...	...	...	...	...	10.2 <sup>13</sup>	16.3	14.4	12.0	12.1
HD9	72	10.0	...	...	...	...	...	19.2 <sup>14</sup>	...	17.8	23.4	17.5

See table 4 for description of footnotes.

TABLE 2

*Effect of protein level on the tryptophan requirement of the chick<sup>1</sup>*

DIET	L-TRYPTOPHAN		EXP. 1		EXP. 2	
			Av. gains	gain/feed	Av. gains	gain/feed
	% of protein	% of diet	gm		gm	
A (10% protein)	0.6	0.06			28	0.13
	0.7	0.07	52	0.23	40	0.20
	0.8	0.08			50	0.22
	0.9	0.09	64	0.26	58	0.24
	1.0	0.10			60	0.25
	1.1	0.11	66	0.27	62	0.27
	1.3	0.13	52	0.24		
	1.7	0.17	58	0.24		
	2.1	0.21	64	0.27		
		0.55	0.11			54
B (20% protein)	0.65	0.13	86 (74) <sup>2</sup>	0.38 (0.39) <sup>2</sup>	90	0.42
	0.75	0.15			112	0.49
	0.85	0.17	115 (117)	0.47 (0.48)	110	0.49
	0.95	0.19			107	0.49
	1.05	0.21	108 (116)	0.45 (0.47)	103	0.48
	1.25	0.25	116 (107)	0.48 (0.47)		
	1.45	0.29	117 (112)	0.48 (0.49)		
	1.65	0.33	107 (102)	0.46 (0.42)		
C (30% protein)	0.37	0.11			22	0.18
	0.43	0.13	44	0.29	48	0.32
	0.50	0.15			63	0.38
	0.57	0.17	92	0.45	82	0.45
	0.63	0.19			100	0.51
	0.70	0.21	96	0.48	106	0.52
	0.83	0.25	96	0.46		
	1.03	0.31	98	0.46		
	1.23	0.37	100	0.47		
		0.32	0.13	32	0.24	28 <sup>3</sup>
D (40% protein)	0.37	0.15			42 <sup>3</sup>	0.33
	0.42	0.17	62	0.39	69 <sup>4</sup>	
	0.47	0.19			70 <sup>3</sup>	0.41
	0.52	0.21	91	0.46	80 <sup>3</sup>	0.46
	0.57	0.23			80 <sup>4</sup>	
	0.62	0.25	82	0.48		
	0.82	0.33	73	0.42		
	1.00	0.40	82	0.45		

<sup>1</sup> Averages of 10 chicks, except if indicated otherwise, during 10-day experimental period.<sup>2</sup> Controls not receiving penicillin in parentheses.<sup>3</sup> Averages of 8 chicks.<sup>4</sup> Averages of 4 chicks.

*Effects of withdrawal of folic acid, calcium pantothenate, pyridoxine and vitamin B<sub>12</sub> from the vitamin supplement*

As clinical signs of pellagra had not appeared in any of the subjects by the 62nd week, folic acid, calcium pantothenate, pyridoxine and vitamin B<sub>12</sub> were removed at this time from the vitamin supplement of 6 of the subjects who were not receiving any lactalbumin. This was done to determine whether these vitamins were preventing the development of a pellagra syndrome. These subjects (U5-100, U6-100, R3-100, R4-100, R8-110 and NR5-100) were chosen for observation of possible effects of prolonged feeding of the basal diet because they had not shown any evidence of liver dysfunction, as determined by bromsulfalein retention test, during the course of the project and therefore had not received any protein supplements. During the following 25 weeks, these subjects showed no significant alterations in their previous clinical or biochemical patterns. It should be noted that two subjects (U6-100 and R4-100) received the basal 100% diet (5.6 mg of niacin and 265 mg% tryptophan) for 87 weeks without any symptoms of pellagra becoming evident. Two other subjects (U5-100 and R3-100) received the basal diet for the same 87-week period except that 7 gm of lactalbumin replaced 7 gm of zein in their diet from the 52nd to 61st week.

*Comparison of increases in N<sup>1</sup>-methylnicotinamide excretion produced by tryptophan and by niacin*

The importance of evaluating tryptophan as a substitute or supplement for niacin should be apparent as any estimation of the niacin equivalence of a given diet must depend upon the amount of tryptophan in the protein supplied (Horwitt, '55). Estimations of how much tryptophan is convertible to niacin and its derivatives depend largely upon the study of the three main excretory products of niacin-tryptophan metabolism, N<sup>1</sup>-Me, the 6-pyridone of N<sup>1</sup>-Me, and quinolinic acid. Analysis for 6-pyr can give useful information when relatively large amounts of N<sup>1</sup>-Me are excreted, but when the levels

percentage of dietary protein, the tryptophan requirement appears to decrease at a decreasing rate.

When a maintenance requirement for tryptophan is estimated and subtracted from the figures obtained for the 10 and 20% protein levels, the remaining amounts of tryptophan required for growth parallel the gains, being in both cases approximately twice as large for the 20 as for the 10% level. It is not so simple to explain the increased requirement at the supernormal protein levels, i.e., where an increase of protein did not result in increased growth.

Some consideration has to be given to the occurrence of more efficient feed utilization at protein levels above those required for optimum growth. A greater gain/feed ratio at equal gains is the result of decreased feed intake of all nutrients, including the amino acids. To maintain a certain absolute amount of tryptophan intake, a more efficient diet would therefore have to contain a higher percentage of all nutrients. Most nutrients will be present in sufficient excess to allow for this slight increase, but a nutrient that is limiting and fed at graded levels might conceivably be shown to be required in larger amounts. It seems, however, that this factor could not explain more than a small part of the increased requirement at supernormal levels.

When a protein deficient in an amino acid is fed, the utilization of this protein is limited by the extent of the deficiency. It is possible that the organism in its attempt to remove the excess amino acids also loses a certain amount of the limiting amino acid. A similar situation is created when excess amino acids, due to a supernormal protein level, have to be removed from the body. The increased dietary requirement for the limiting amino acid might be an expression of these losses. It remains to be determined where these losses occur.

Sauberlich and Salmon ('55) have shown with rats that an imbalance of tryptophan created by the addition of protein sources deficient in this amino acid does not result in lowered digestibility and absorption of the protein. They observed an actual increase of amino acids in the urine, including an

1:65. In subsequent weeks, there were too few subjects not receiving extra protein to warrant such calculations.

From such data as presented above, a factor of 1:60 has been chosen to designate the relative efficacy of tryptophan in substituting for niacin. It is the opinion of the senior author, after attempting to analyze the data from each subject that, though there are large individual variations, a factor of 1:60 is a logical starting point for future studies. Past experience with "load tests" with other vitamins has shown that the proportions of a vitamin or its products excreted after ingestion are dependent upon the nutritional state of the subject being tested. Thus, the administration of a vitamin supplement equivalent to one day's requirement would, if given to a deficient subject, produce little urinary excretion of the given vitamin or its products. Therefore, since the calculations above compare groups in different stages of niacin saturation, one can predict that a larger proportion of the administered niacin would be excreted by the NR group than by the TR group which would produce an apparent ratio that would make tryptophan seem less effective than niacin. In other words, the 1:60 ratio includes a considerable margin of safety; less tryptophan than suggested by the ratio of 1:60 would probably be needed to replace each milligram of niacin in the non-growing adult.

The data discussed in the section on the effects of lactalbumin supplementation on  $N^1$ -Me excretion, though meager, do not show any marked differences between the effects of tryptophan fed as a component of lactalbumin and of L-tryptophan fed as an isolated amino acid.

#### DISCUSSION OF NIACIN RATIOS

Analysis of the data reported should take into account the results of the Tulane studies (Goldsmith et al., '52) some of which were conducted at the same time as the Elgin project. These workers produced niacin deficiency in three subjects in 50 days on a corn diet which contained about 4.7 mg of niacin and 190 mg of tryptophan. On the other

little work has been done with graded levels close enough to each other to establish the actual minimum requirement. Also, the use of the racemic mixture with various assumptions concerning the availability to the chick of the *D* isomer, and the use of higher protein levels might sometimes cause requirements to appear higher than they really are.

In contrast with methionine, considerable excess of dietary tryptophan above the requirement for optimum growth did not seem to exert a growth-depressing effect on the chicks. This is in agreement with observations made by Fisher et al. ('54b) and by other authors.

It is of interest to observe that work done by Salmon ('54) showed that the tryptophan requirement of the rat, with niacin provided at adequate levels, increases with increasing protein levels but at a slower rate. These results closely parallel those obtained in the present investigation with chicks.

#### SUMMARY

The dietary tryptophan requirement of growing male cross-bred chicks has been shown to increase with increasing protein levels, though at a slower rate than the latter. When the diet contained 10, 20, 30 or 40% protein, the minimum requirement for tryptophan was estimated to be 0.09, 0.143, 0.182, and 0.20% of the diet respectively. Thus, a protein causing a slight tryptophan deficiency when incorporated into a diet at the 20% level might conceivably supply sufficient tryptophan for optimum growth when incorporated into the diet at a higher level.

Supplementation with an antibiotic did not appear to have a sparing effect on the requirement for dietary tryptophan.

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TABLE 8

*Niacin ratios of experimental diets*

DIET	CALORIES	NIACIN mg	TRYPTOPHAN mg	NIACIN EQUIVALENTS	NIACIN RATIO (niacin equivalents per 1000 Cal.) <sup>1</sup>	PROPORTION WITH PELLAGRA SYMPTOMS <sup>2</sup>
Tulane "corn" <sup>3</sup>	1700-2100 <sup>4</sup>	4.4-4.6	177-195	7.4-7.8	(3.7-3.9)	3/3
Tulane "wheat" <sup>4</sup>	1600-1900	4.2-4.7	177-193	7.3-7.8	(3.7-3.9)	2/3
Goldberger <sup>5</sup>	3000	6.7	330	12.2	4.1	6/11
Tulane "wheat" <sup>4</sup>	1750	4.9	200	8.2	(4.1)	1/2
Elgin (90%)	2070	5.2	238	9.2	4.4	0/1
Elgin (100%)	2300	5.8	265	10.2	4.4	0/11
Elgin (110%)	2530	6.4	292	11.3	4.4	0/1
Elgin (120%)	2760	7.0	318	12.3	4.4	0/2
Tulane "corn" + niacin <sup>6</sup>	1970	6.7	190	9.9	4.9	0/1

<sup>1</sup> Diets providing less than 2000 calories are arbitrarily calculated as containing 2000 calories on assumption that there is a minimum basal requirement for niacin-tryptophan of approximately 8.8 niacin equivalents and that increased needs for greater caloric consumption are measurable above this level as a multiple of 1000 calories (see text, pp. 35, 36).

<sup>2</sup> Calculation of data presented in a recent summary of the Tulane experiments (Goldsmith, personal communication) shows that symptoms of pellagra were obtained in 13 out of 15 individual subject trials when 8.7, or less, niacin equivalents were fed. Of these 15 subjects, symptoms were obtained in three out of 5 on wheat diets, 4 on U. S. corn, three on lime-treated Guatemalan corn, and two on untreated Guatemalan corn.

<sup>3</sup> Goldsmith et al. ('52).

<sup>4</sup> Goldsmith, personal communication.

<sup>5</sup> Calculated from Frazier and Friedemann ('46).

<sup>6</sup> Same diet as "Tulane corn" above, plus 2 mg of niacinamide per day.

# VITAMIN B<sub>12</sub> CONTENT OF MILK AND MILK PRODUCTS AS DETERMINED BY RAT ASSAY

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Practically all of the values reported in the literature for the vitamin B<sub>12</sub> content of cow's milk have been determined by microbiological assay. Moreover, relatively few figures are available for the B<sub>12</sub> potency of individual milk products as estimated by any method.

The usefulness of animal assays both for comparison with other methods of determination and as some indication of the physiological vitamin value of foods is generally recognized. In the present paper values are given for the vitamin B<sub>12</sub> potency of milk and some milk products as determined by rat assay. Possible effects of breed and ration of the cows and of various methods of handling the milk are also considered. The method used employed non-hyperthyroid B<sub>12</sub>-deficient rats (Cary et al., '46; Hartman, '46). The basal ration, containing lactose and casein, was designed especially for determining the vitamin B<sub>12</sub> content of dairy products. Comparison of this procedure with other rat-growth methods, as applied to milk products, will be considered in another communication.

## ASSAY METHOD

The rats used were 28-day-old males weaned from stock colony<sup>1</sup> mothers fed a B<sub>12</sub>-deficient ration during lactation.

<sup>1</sup>For composition of stock ration, see Hartman et al. ('51).



estimating the "niacin ratio" of a diet, one never uses less than 2000 calories in the calculation even though the subject in question is consuming less than this amount. This eliminates the possibility of having an apparently adequate value of this ratio in a subject consuming only a small part of a well-balanced diet. In effect, the amount adequate for 2000 calories would be the minimum basal requirement for the adult.

An alternate calculation of the minimum niacin-tryptophan requirements of man might be made by subtracting a minimum figure for tryptophan requirements (Rose, '49) for maintenance (about 150 mg or 2.5 niacin equivalents) and then estimating the remaining niacin equivalents. However, since less than 200 mg of tryptophan are found only in experimental diets or in areas of severe starvation, no practical point is served by treating the maintenance requirement separately. The niacin ratio, if used as suggested, tends to correct for differences in the size of the individual, as size, protein and basal caloric needs are all related although it is recognized that the tryptophan needs are only indirectly related to caloric intake.

Table 9 lists the niacin ratios of some representative foods to show how relatively unimportant can be the niacin content of good sources of tryptophan. This table also demonstrates how corn itself can vary. To obtain the data on corn, calculations were based on the niacin and tryptophan contents of 23 different varieties of Guatemalan corn as reported by Aguirre et al. ('53). The 4 Guatemalan corns chosen in table 9 are those which contained the lowest niacin, the lowest tryptophan, the highest niacin and the highest tryptophan contents, respectively. In order to estimate the caloric content of these corns, the results published by Bressani et al. ('53) were calculated from their data on fat, nitrogen, crude fiber and ash. The average niacin ratio of the Guatemalan corns is about 6.7 and pellagra is relatively uncommon in Central America where it is claimed that the average Indian consumes about 500 gm of corn per day. This is considered to provide

separate supplements, the B<sub>12</sub> by syringe and the milk in small glass dishes. For the assay of milk products, the reference B<sub>12</sub> and the test supplement were incorporated at given levels in the B<sub>12</sub>-deficient assay ration; the test material replaced equal amounts of protein (casein), lactose, salts and fat. The resulting diets were fed ad libitum.

To ascertain the character of the dose-growth response curve, results obtained with the reference B<sub>12</sub> were utilized.

TABLE 1  
*Dose-growth regression lines<sup>1</sup> with crystalline vitamin B<sub>12</sub>*

TYPE OF REGRESSION LINE	SERIES 1 B <sub>12</sub> FED AS SEPARATE DOSES <sup>2</sup>			SERIES 2 B <sub>12</sub> FED AT GIVEN LEVELS IN RATION <sup>3</sup>		
	Av. slope	Coeff. of variation of av. slope	Combined slope	Av. slope	Coeff. of variation of av. slope	Combined slope
		%			%	
Growth <sup>4</sup> -log dose	47.6	5.6	46.1 <sup>5</sup>	53.6	29.0	....
Log growth <sup>4</sup> -log dose	0.601	34.6	...	0.706	17.3	0.681

<sup>1</sup> The intercept of the regression line of any given assay was calculated from the internal data of that assay.

<sup>2</sup> Five assays; per assay, 8 to 19 litters, 2 to 4 doses; range of doses over all assays, 0.01 to 0.50 µg/day.

<sup>3</sup> Seven assays; per assay, 7 to 14 litters, 2 to 3 levels; range of levels over all assays, 0.005 to 0.06 µg/10 gm ration.

<sup>4</sup> Growth = av. 4 weeks weight gain of dosed rats minus av. 4 weeks weight gain of negative controls; range of these growths: 9 to 85 gm in the separate dose assays, 11 to 70 gm in the level-in-ration assays; range of av. weight gain of negative controls, 52 to 82 gm.

<sup>5</sup> Standard error:  $\pm 2.18$ .

The assays in which the supplements were fed separately from the ration have been designated as series 1. Within individual assays as well as over this whole series (table 1), the results were found to fit quite well a linear regression of weight gain on log dose. Those assays in which the supplements were incorporated at given levels in the ration have been designated as series 2. In contrast to series 1, the data from series 2 fitted better a log growth-log dose linear re-

the Guatemalan diet were to be replaced by "civilized" forms of carbohydrate, such as corn syrup, corn starch or sucrose, pellagra would be common in that country.

The statements made, herein, are not intended to eliminate the possibility that some corn products may have such low "niacin ratios," or such low niacin-tryptophan availability, that their consumption, exclusively, would promote pellagra. Rather it is an attempt to focus more attention on a simple relationship of the niacin-tryptophan content of the diet in terms of total calories consumed. Justification for using calories as a base line, rather than body weight, may be obtained from comparisons of the Goldberger, Tulane and Elgin diets and from experience with animals where niacin and thiamine needs were directly related. The Goldberger prison diet (Goldberger and Wheeler, '20) provided relatively high amounts of niacin and tryptophan but the caloric intake was also high. The prisoners had work to perform, whereas the Tulane and Elgin subjects had a minimum amount of activity. Krehl et al. ('46) have pointed out that their estimations of the tryptophan requirement of the rat was considerably less than the level of 0.2% suggested by Rose ('37) who used a diet relatively high in fat and calories.

The data which suggest that L-lysine supplementation (1 gm per day) had a deleterious effect on the formation of N<sup>1</sup>-Me reinforce reports (Koepppe and Henderson, '55; Hanks et al., '49) that amino acids in the diet must be properly balanced.

When the diet of the TR group (about 9% protein) was fed to weanling rats (Horwitt, '53) their growth rate averaged less than 1.3 gm per day and fatty livers were produced. Adult men were reasonably well maintained on the same diet although some evidence of liver dysfunction was obtained. The addition of lactalbumin to this diet produced normal rats with a growth rate of 4.3 gm per day. These observations should be considered in the light of the increased protein requirements for rapid growth, and the high frequency of kwashiorkor in Guatemalan children on corn diets which do not produce pellagra.

crude casein and dried skim milk also failed to give additional growth over that obtained with maximally effective levels of vitamin B<sub>12</sub>.

In each assay one to three doses or levels of the reference B<sub>12</sub> and one or two of the unknown were used. Separately fed supplements of the milk and of the vitamin B<sub>12</sub> were given 4 or 5 times a week but the amounts have been expressed as the quantities received per day for a 6-day week.

#### RESULTS AND DISCUSSION

Considerable variation has been found (Collins et al., '51, '53) in the vitamin B<sub>12</sub> level in milk between cows of the same breed and between samples from the same animal taken at different times. Therefore it should be emphasized here that the various lots of milk used in the present work represent herd milk. Moreover, a given lot consisted of a series of samples collected three, 4 or 5 times per week throughout the assay, each sample representing mixed milk from a number of cows in various stages of lactation.<sup>8</sup>

A previous report from this laboratory (Hartman and Dryden, '52) showed that cobalt, added to cow rations already containing amounts of this element adequate for normal health and functioning, failed to increase the vitamin B<sub>12</sub> content of the milk as determined by the present rat assay method. The results of studies on the possible effects of other factors on the vitamin B<sub>12</sub> potency of milk are summarized in table 2.

It can be seen (table 2, experiment 1) that the vitamin B<sub>12</sub> potency of raw Jersey and Holstein milk produced by cows on pasture was not significantly different from that produced by cows fed only barn rations. This result is in accord with findings of preliminary comparative rat growth studies (Hartman et al., '49) carried out before crystalline vitamin B<sub>12</sub>

<sup>8</sup> These samples were collected from groups of cows in the dairy herd at the Beltsville Agricultural Research Center. The average number of cows per sample ranged from 4 to approximately 60, while the total number of cows used per assay varied from 8 to more than 60.

Urinary excretions of niacin and tryptophan were not directly related to the usual levels at which these were present in the diet.

Data pertaining to the possibility that lysine or methionine added as supplements may inhibit the formation of niacin metabolites are presented and discussed.

While attempting to correlate the effects of riboflavin deficiency on "pellagrigenic" diets the following observations were made; (1) That a diet low in niacin and tryptophan did not cause any noticeable alterations in the previously reported course of ariboflavinosis; (2) that supplementation with vitamin B<sub>12</sub> produced no changes in either the development or repair of ariboflavinosis and (3) confirmed a previous report that scrotal dermatitis was a consistent observation in ariboflavinosis in man.

The withdrawal of folic acid, calcium pantothenate, pyridoxine, and vitamin B<sub>12</sub> as supplements to the basal diet of 5 subjects for a period of 25 weeks had no noticeable effect on the subjects involved. One may assume that either the basal diet was not grossly deficient in these vitamins or that the previous 62 weeks of supplementation had created a large reserve.

The level of excretion of N<sup>1</sup>-methylnicotinamide has been confirmed as an indicator of the ability of the organism to extract niacin derivatives from the diet and in this manner may prove to be a useful index of niacin-tryptophan availability.

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became available as an assay standard. These earlier studies indicated no marked difference, if any, in the vitamin B<sub>12</sub> potency of milk from cows on pasture, from cows on barn feeds or from cows which had been for several years on a ration in which no vitamin B<sub>12</sub> activity could be detected. On the other hand, de Heus and de Man ('51) and van Koetsveld ('53) found the vitamin B<sub>12</sub> content to be about twice as much early in the pasture feeding period as it was previously during indoor feeding; the former workers also observed a gradual decline to the indoor level. The results obtained in the present studies are not necessarily in conflict with those obtained by the above workers since the cows used here had been on pasture for at least one and a half months before collection of the first sample.

Milk of the Jersey and Holstein breeds was compared in two assays (table 2, experiment 2). In one case, the cows of both breeds were on pasture. In the other, they were on barn feeds. In neither instance was there a significant difference in vitamin B<sub>12</sub> potency. These findings are at variance with the conclusion of Anthony et al. ('51) that Holstein milk showed a greater concentration of B<sub>12</sub> than Jersey milk. They are, however, in accord with the observations of the Wisconsin workers (Collins et al., '51) who found no noticeable difference between Jersey, Holstein and Guernsey milk and who came to no contrary conclusion in further studies (Collins et al., '53). Sreenivasamurthy et al. ('50, '53) apparently found no marked differences in the vitamin B<sub>12</sub> activity of milk from several breeds of Indian cows.

In some of the above assays, portions of samples were, where necessary, stored at about 0°C. for feeding on days intervening between collections. From experiment 3, it can be seen that storage of raw milk in a household-type refrigerator at this temperature for one, two or three days brought about no detectable change in vitamin B<sub>12</sub> potency.

Direct comparison was made in 5 assays between raw and pasteurized fractions of each sample collected (experiment 4). Although in every assay the pasteurized milk was on the

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ent organisms, *B. coli*, *Ochromonas malhamensis* and *L. leichmannii* ATCC 4797, Gregory ('54) obtained approximately the same results (1.8, 2.3 and 2.9 µg/l, respectively) for the vitamin B<sub>12</sub> content of a single sample of milk. Most of the literature values have been determined with *L. leichmannii*

TABLE 3  
Vitamin B<sub>12</sub> content of certain milk products

PRODUCT	NO. OF SAMPLES	NO. OF BRANDS	TYPE OF ASSAY	VITAMIN B <sub>12</sub> CONTENT  µg/kg	REFERENCE
Dried whole milk	2	2	Normal rat	36,39	Present studies
	1		Hyperthyroid rat	25	Lewis et al. ('49)
	1 <sup>†</sup>		<i>L. leichmannii</i>	20	Elvehjem ('50)
Dried skim milk	3	2	Normal rat	37,39,42	Present studies
	1		Chick	60	Lillie et al. ('54)
	1 <sup>‡</sup>		<i>L. leichmannii</i> 4797	30	Lillie et al. ('54)
	1 <sup>†</sup>		<i>L. leichmannii</i> 4797	30	de Heus and de Man ('51)
Casein, crude	2	1	Normal rat	64,70	Present studies
	1		Hyperthyroid rat	30	Lewis et al. ('49)
	2 <sup>†</sup>		<i>L. leichmannii</i>	30,70	Elvehjem ('50)
	11		<i>L. leichmannii</i> 4797	90 <sup>‡</sup>	de Heus and de Man ('51)
	1 <sup>†</sup>		<i>L. leichmannii</i> 4797	104	Peeler et al. ('51)
Dried whey	2 <sup>‡</sup>	2	Normal rat	11,33	Present studies
	3		Chick	20,30,30	Lillie et al. ('54)
	2 <sup>†</sup>		<i>L. leichmannii</i> 4797	10,30	Lillie et al. ('54)

<sup>†</sup> Same sample as assayed with the chick.

<sup>‡</sup> According to the authors, calculated from average value for casein fraction prepared from fluid whole milk.

<sup>‡</sup> From Cheddar cheese.

<sup>†</sup> Same samples which yielded 30 and 20, respectively, by chick assay.

ATCC 4797. The following average figures have been obtained (µg/l): 5.6, 5.9 and 7.6 (Anthony et al., '51); 3.1,<sup>10</sup> 3.4, 4.1<sup>10</sup> and 6.6 (Collins et al., '51, '53); 3 (Ford et al., '53); 4 (Gregory et al., '52); 3.8 (de Heus and de Man, '51); 4.3<sup>10</sup> (Karlin, '54); 6.3 (van Koetsveld, '53); 1.8, 1.7 and 4.1 (Rusoff and Haq, '54). Although comparative data are not presented,





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# COMPARATIVE ASSAY FOR VITAMIN B<sub>12</sub> IN CERTAIN MILK PRODUCTS BY VARIOUS RAT GROWTH METHODS

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Rat growth assay methods for the determination of the vitamin B<sub>12</sub> potency of natural materials involve the use of either the normal rat or the hyperthyroid rat. In the first instance, weanling young, which have been depleted by feeding their mothers a B<sub>12</sub>-deficient diet, are themselves fed a similar diet during the assay period (Cary et al., '46; Hartman, '46; Zucker et al., '48, '50; Cuthbertson and Thornton, '52; Sherman et al., '55; Hartman et al., '56). In the second type of assay, stock colony young are made B<sub>12</sub>-deficient subsequent to weaning by inclusion of a thyroactive substance in a B<sub>12</sub>-deficient ration and are then continued on the same ration during the assay period (Register et al., '49a, b; Emerson, '49; Frost et al., '49, '53; Tappan et al., '50; Cheng and Thomas, '51; Scheid et al., '52).

Assay methods also vary in the manner of substitution of the test material in the assay ration and in the composition of the assay ration itself in respects other than the presence or absence of thyroactive material. The more important of such variations in the assay ration concern the sources of carbohydrate and of protein. The protein may be furnished by animal protein in the form of casein or by plant protein from such sources as soybean meal, corn meal, cottonseed meal, or the like.

'51; Sreebny and Nikiforuk, '51; Nikiforuk and Sreebny, '53; and Hunter and Nikiforuk, '54). In this present series of three experiments with white rats, we have studied the effect of supplements of ethylene diamine tetraacetic acid (EDTA) to various diets under different circumstances as a means to define the nature of these influences *in vivo*.

TABLE 1  
*Composition of diets*

INGREDIENTS	RATION 700	RATION 770	RATION 700 + 0.2% EDTA	RATION 700 + 0.4% EDTA	RATION 770 + 0.2% EDTA	RATION 770 + 0.4% EDTA
	gm	gm	gm	gm	gm	gm
Sucrose	670		670	670		
Lard		120			120	120
Casein <sup>1</sup> with added B-complex vitamins <sup>2</sup>	240	240	240	240	240	240
Casein <sup>1</sup>		400			400	400
Corn oil with added vitamins A, D, E, K <sup>2</sup>	50	50	50	50	50	50
Salt mixture <sup>2</sup>	40	40	40	40	40	40
Whole liver extract (1: 20)		20			20	20
Desiccated liver	40	20	40	40	20	20
Ethylene diamine tetra- acetic acid (EDTA) <sup>2</sup>			2	4	2	4

<sup>1</sup> Borden's crude casein.

<sup>2</sup> J. Dent. Res., 26: 47 (1947).

<sup>3</sup> Purchased under trade name Sequestrene, Alrose Chemical Co.

#### EXPERIMENTAL

The composition of the 6 diets used in these experiments is given in table 1. The experimental plans are listed in the first three columns of table 2. In all cases, weanling littermates were distributed with respect to weight and sex as evenly as possible among the groups of an experiment. The rats were caged in individual wire-bottom cages with their own food

sition: sucrose, 19.12; cottonseed meal,<sup>4</sup> 69.46; DL-methionine, 0.40; lysine, 0.40; salt mixture,<sup>5</sup> 2.95; cottonseed oil, 7.19; fish liver oil,<sup>2</sup> 0.11; and added vitamins,<sup>3</sup> 0.37. The corn-soy ration used in experiments in table 2 and for some of the assays in table 3 had the following percentage composition: yellow corn meal, 42.42; soybean meal, 42.42; DL-methionine, 0.30; salt mixture (Hawk and Oser, '31), 4.50; cottonseed oil, 9.85; fish liver oil,<sup>2</sup> 0.15; and added vitamins,<sup>3</sup> 0.37. Modifications of these basal rations are indicated in the appropriate places in the paper.

Vitamin B<sub>12</sub><sup>6</sup> and iodinated casein,<sup>7</sup> where fed, were incorporated in the basal rations in the amounts indicated in the tables.

## RESULTS AND DISCUSSION

### *Tests of the suitability of certain assay rations for measuring the vitamin B<sub>12</sub> activity of milk products*

Before carrying out comparative assays of the vitamin B<sub>12</sub> activity in milk products, preliminary experiments were run to determine whether rats fed the various rations under consideration would respond only to this vitamin. It seemed possible that the assay rations might be deficient in unidentified nutrients contained in the test substances or, on the other hand, that some component of the test materials might exert a depressing effect on growth. As an example of the latter effect, Ott ('51) found that substituting dried whey or vitamin-free casein for cerelese in a B<sub>12</sub>-assay ration for chicks led to a

<sup>4</sup> Screw-pressed meal of low free gossypol content; kindly furnished by the Engineering and Development Division, Southern Regional Research Laboratory, U. S. Department of Agriculture, New Orleans, La.

<sup>5</sup> Hawk and Oser, '31, modified to allow for the ash content of cottonseed meal. Such modification consisted of removing the potassium phosphate, magnesium carbonate, and magnesium sulphate, decreasing by one half the relative amounts of potassium chloride and calcium carbonate and increasing by about 64% the sodium chloride.

<sup>6</sup> Kindly supplied by Merck and Co., Inc., Rahway, N. J.

<sup>7</sup> Kindly supplied by Cerophyl Laboratories, Inc., Kansas City, Mo.

cups and water bottles. They were housed in air-conditioned, temperature- and humidity-controlled rooms. At the end of each experiment, the heads were fixed in 95% alcohol for 48 hours, and then skinned. The molar teeth were examined and the lesions in the occlusal sulci evaluated under a binocular microscope ( $\times 30$ ) by grinding successive planes of the teeth with the help of a running grinding stone, by the method of Shaw et al. ('44).

The first experiment was designed to determine the influence of two levels of EDTA on the initiation and progression of carious lesions in the occlusal fissures and also upon the initiation of any lesions on the smooth surfaces. A comparison of the influence of a high-carbohydrate diet with that of a carbohydrate-free diet was included. The second experiment was conducted to test the influence of sialoadenectomy upon the initiation of EDTA-induced lesions. In both of these experiments, the subjects used were representatives of a relatively highly caries-susceptible strain. The objectives of the third experiment were two-fold: first, to determine the influence of EDTA in a highly caries-resistant strain of rodents and second, to test its influence when introduced into the stomach by a tube to prevent contact with the oral tissues. The basal diet for all groups in this experiment was no. 700. The subjects in the first group received no supplement while those in the second group were given 0.4% EDTA in the diet. The amount of ration consumed by each rat in the second group was determined every 24 hours and the amount of EDTA consumed by each rat calculated. This amount of EDTA in suspension was given by stomach tube to the respective littermates in the third group by the procedure described by Kite et al. ('50).

## RESULTS

In addition to the carious lesions that are normally found in the occlusal sulci of our caries-susceptible strain of rats, the EDTA-fed rats maintained on high-carbohydrate diet 700 had a different type of lesion on the smooth surfaces. The

TABLE 1

*Effect of incorporating certain milk products<sup>1</sup> into rations containing a maximally effective amount of vitamin B<sub>12</sub>*

EXP.	NO. OF LITTERS	IODINATED CASEIN	VITAMIN B <sub>12</sub>	AVERAGE WEIGHT GAIN IN FOUR WEEKS				
				Without milk product	With milk product		F <sup>4</sup>	Least signif. diff.
					Not adjusted <sup>2</sup>	Adjusted <sup>2</sup>		
		%	µg/10 gm ration	gm	gm	gm		gm
<i>Basal ration containing alcohol-extracted casein:</i>								
<i>Dried skim milk:</i>								
			0.5	165	157	165		
1	20	0.00	1.5	164	165	...	1.2	...
			0.5	121	114	121		
2	6	0.15	1.5	134	110	...	3.9*	14.1
<i>Crude casein:</i>								
3	6	0.00	0.5	165	...	164	.. <sup>5</sup>	...
<i>Basal ration containing soy protein<sup>3,7</sup>:</i>								
<i>Dried skim milk:</i>								
4	16	0.00	1.0	195	...	184	4.6*	10.8
5	4	0.20	1.0	160	...	122	15.1*	30.3
<i>Basal ration containing cottonseed meal<sup>3,7</sup>:</i>								
<i>Dried skim milk:</i>								
6 <sup>9</sup>	8	0.00	1.0	145	138	...	1.3	...
7	7	0.25	2.0	161	135	...	17.4**	15.0
<i>Crude casein:</i>								
8	7	0.00	2.0	167	154	...	2.9	...
9	7	0.25	2.0	161	143	...	22.2**	9.1

<sup>1</sup> Fed at level of 10% in experiments 1, 2, 3, 6 and 7, 19% in experiments 8 and 9, and 25% in experiments 4 and 5.

<sup>2</sup> Replaced an equal amount of carbohydrate (dextrin in experiments 1 and 2), except in experiment 6, where it replaced an equal amount of the whole ration.

<sup>3</sup> Replaced an equivalent amount of carbohydrate (lactose in experiments 1 and 2), salts, fat and B<sub>12</sub>-deficient casein.

<sup>4</sup> The symbol \*\* adjacent to or in connection with a F value indicates statistical significance at or less than the 1% level; \* indicates significance at the 5% level or between the 5% and 1% levels; no \* indicates no statistically significant difference.

<sup>5</sup> Treatment mean square less than error mean square; therefore not significant.

<sup>6</sup> Modified to contain half the quantity of added vitamins. Half the litters in experiment 4 received rations containing 1% sulfasuxidine.

<sup>7</sup> Mothers of experimental rats were fed similar rations during lactation. All mothers received 1 µg vitamin B<sub>12</sub> per 10 gm ration.

<sup>8</sup> In experiment 6, the ration was modified at the expense of sucrose to contain 50.86% cottonseed meal and 6.7% cottonseed oil.

<sup>9</sup> Dried whole milk fed instead of dried skim milk.



In many instances among the EDTA-fed rats, diffuse, whitish borders were observed around the margin of the carious lesions in the occlusal fissures. In many ways these borders strongly resembled the smooth-surface lesions and were distinctly dissimilar to the borders of the occlusal lesions in rats fed the basal ration 700. Yet at the same time these borders were sufficiently small and in such early stages of involvement that they did not seem indicative of more rapid progression in the occlusal lesions.

The average number of molars with carious lesions in the occlusal sulci, the average number of these carious lesions and the average extent of these carious lesions and the frequency and extent of decalcified lesions on the smooth surfaces among the animals in the three experiments are presented in table 2. The rats in group 1, of the first experiment which were on ration 700, had an incidence of dental caries that was typical of intact representatives of this strain of caries-susceptible rats. The values for occlusal caries in the EDTA-fed rats of groups 2 and 3 had a tendency to be slightly higher than for the controls in group 1; however, there was no striking statistical significance to these minor increases. As would be expected from previous experiments in this laboratory with carbohydrate-free diets, none of the rats in groups 4 through 6 developed any carious lesions on the occlusal surfaces.

No lesions were observed on the smooth surfaces of the teeth of the rats in group 1. The molars of the rats in group 2 had a relatively high incidence of smooth surface lesions that were in various early stages of development. These lesions were relatively early ones that consisted of white, opaque, diffuse streaks on the buccal side of the teeth along the gingiva. Some of the streaks were soft, others were still hard. The molars of the rats in group 3 had a greater num-

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#### DESCRIPTION OF FIGURES

Figs. 1 to 4 Photographs of fixed preparations of white rat mandibles that were selected to illustrate the types and stages of smooth-surface lesions observed in EDTA-fed subjects. Mag.  $\times 18$ . Immediately above each figure are the individual scores for the lesions.

even though the lot of skim milk powder which was used had been found by our regular assay method to contain considerable vitamin B<sub>12</sub>. Part of the failure of the rats to respond with increased growth was evidently due to the lactose content of the skim milk powder (experiment 3). In three experiments, B<sub>12</sub>-active crude casein gave somewhat variable re-

TABLE 2  
*Effect of incorporating certain milk products<sup>1</sup> into B<sub>12</sub>-deficient plant protein rations*

EXP.	NO. OF LITTERS	TYPE OF BASAL RATION <sup>2</sup>	AVERAGE WEIGHT GAIN IN THREE WEEKS				F <sup>4</sup>	Least signif. diff.
			No vitamin B <sub>12</sub>		Vitamin B <sub>12</sub> <sup>3</sup>			
			Without milk product	With milk product	Without milk product	With milk product		
			gm	gm	gm	gm		
<i>Dried skim milk:</i>								
1	5	Soy protein	54	54	77	73	6.0**	15.4
2	8	Corn-soy	41	61	93	86	25.4**	13.5
<i>Lactose:</i>								
3	8	Soy protein	66	44	91	94	34.9**	11.8
<i>Crude casein:</i>								
4	8	Soy protein	60	68	104	101	33.7**	11.5
5	6	Soy protein <sup>5</sup>	65	74	114	106	12.0**	20.7
6	8	Soy protein	65, 54 <sup>6</sup> , 56 <sup>7</sup>	83	..	..	19.1**	9.1
7	6	Corn-soy	60	77	77	84	4.2*	15.0
8	7	Corn-soy	86	113	121	122	12.0**	14.2

<sup>1</sup> Ten per cent included in ration. With soy protein rations, replaced an equal amount of carbohydrate; with corn-soy rations, replaced 5% yellow corn meal and 5% soybean meal.

<sup>2</sup> Soy protein ration modified here to contain 53.23% sucrose and 31.70% soy protein. The rations in experiment 8 contained no iodinated casein, whereas all the others contained 0.15% of this product during the experimental period. Mothers of experimental rats were fed a B<sub>12</sub>-deficient purified casein-sucrose ration during lactation.

<sup>3</sup> One microgram in 10 gm ration.

<sup>4</sup> See footnote 4, table 1.

<sup>5</sup> Ration modified to contain dextrin in place of sucrose.

<sup>6</sup> Sufficient additional soy protein substituted for an equal amount of sucrose so that protein content of ration was same as that of test ration containing crude casein.

<sup>7</sup> B<sub>12</sub>-deficient extracted casein and sucrose substituted for soy protein. Protein content (25%) maintained the same as in soy protein basal ration.

ber of smooth surface lesions that were of more advanced stages of development. The extent and the frequency of lesions on the smooth surfaces had increased definitely with the increased concentration of EDTA. However, where EDTA supplements to carbohydrate-free diet 770 were fed to the rats in groups 5 and 6, no smooth surface lesions were caused. In other words, the carbohydrate-free diet, in addition to preventing carious lesions in the occlusal sulci, inhibited the formation of these EDTA-induced lesions on the smooth surfaces.

The molars of the sialoadenectomized rats in groups 4, 5 and 6 of experiment 2 had a much higher incidence of tooth decay than their intact littermates. No lesions were observed on the smooth surfaces of the molars of the intact or sialoadenectomized rats in groups 1 and 4 that were fed only the basal ration. The incidence of smooth surface lesions in the intact rats with EDTA supplements was observed to be very low; however, major and statistically significant increases in smooth surface lesions were observed in the molars of the sialoadenectomized animals.

In experiment 3, the rats in group 1 had an incidence of dental caries that was typical of this strain of caries-resistant rats, when maintained on cariogenic ration 700 for this time interval. In addition, no smooth surface lesions were observed. The rats in group 2 which were on diet 700 plus 0.4% EDTA had a comparable incidence of tooth decay and had a moderate incidence of smooth surface lesions in various stages of development. The three categories of dental decay for the rats in group 3, which were fed diet 700 with supplements of EDTA by stomach tube were slightly but not significantly lower than the scores for the rats in groups 1 and 2. Minor lesions on the smooth surfaces were observed in a few molars as white, opaque, diffuse streaks. These streaks were faint and hard unlike the ones found on the teeth of animals in group 2. Both the incidence and extent of occlusal caries and of lesions on the smooth surfaces were much less frequent in

protein, the presence or absence of iodinated casein, the manner of substitution of the test products in the basal ration and the pre-treatment of assay animals. The results of the assays are given in table 3.

TABLE 3

*Vitamin B<sub>12</sub> potency of one lot of dried skim milk and one lot of crude casein, as determined by various methods of rat growth assay*

GROUP	TYPE OF RATION FED	IODI- NATED CASEIN	DRIED SKIM MILK			CRUDE CASEIN		
			Assay no.	No. of litters	Vit. B <sub>12</sub> potency	Assay no.	No. of litters	Vit. B <sub>12</sub> potency
		%			μg/kg			μg/kg
1	Alcohol-extracted casein basal ration (no. 262):							
	Not adjusted <sup>1</sup>	0.00	1	7	5	12	10	38
	Adjusted <sup>2</sup>	0.00	2	7	38	13	10	71.57 <sup>2</sup>
		0.00	3	9	44	..	..	..
		0.04	4	6	51	14	6	53
		0.04	5	8	38	..	..	..
2	Corn-soy basal ration:							
	Not adjusted <sup>4</sup>	0.05	6 <sup>5</sup>	10	0	15 <sup>5</sup>	10	37
		0.05	7	9	14	16	9	49
		0.15	8	9	10	17	7	36
		0.15	9	5	5	..	..	..
	Adjusted <sup>6</sup>	0.05	10	9	27	18	10	77
		0.05	..	..	..	19	9	61
		0.15	11	3	12	..	..	..

<sup>1</sup> Milk product replaced an equal amount of dextrin.

<sup>2</sup> Skim milk replaced an equivalent amount of lactose, salts and B<sub>12</sub>-deficient casein. Crude casein replaced an equal amount of B<sub>12</sub>-deficient casein.

<sup>3</sup> Values for two different levels.

<sup>4</sup> Half of milk product replaced an equal amount of corn; half replaced an equal amount of soybean meal.

<sup>5</sup> Mothers continued on stock ration after parturition; weanling young depleted of B<sub>12</sub> by feeding them the corn-soy basal ration (with iodinated casein) for two weeks before they were placed on assay. In all other assays, assay rats were weanling young from mothers placed at parturition on a non-thyroactive B<sub>12</sub>-deficient ration (no. 260 in group 1 and a purified casein-sucrose ration in group 2).

<sup>6</sup> In these assays, the basal assay ration fed to the negative controls and to the rats administered the reference standard B<sub>12</sub> was modified as follows: in assays 10 and 11, to contain lactose, salts and B<sub>12</sub>-deficient casein equivalent to that in the test ration; in assay 18, to contain an amount of B<sub>12</sub>-deficient casein equivalent to the amount of crude casein in the test ration; in assay 19, by adjusting the proportions of corn and soybean meal, to have the same protein content as the test ration.

lesions. In addition, supplements of penicillin, terramycin or bacitracin to the diet almost completely prevented the lesions induced by the feeding of EDTA in the diet (Stephan et al., '52; Fitzgerald, '55).

These observations suggest that the production of lesions by EDTA was not a simple decalcification of tooth surfaces. In all cases the production or the prevention of these lesions closely paralleled circumstances that led to the production or the prevention of carious lesions. From the studies with our strains of caries-susceptible and caries-resistant rats, it appeared that the EDTA has acted as an additional factor in the initiation and progression of caries-like lesions in areas where our normal procedures, including the extreme penalizing influence of sialoadenectomy, were to a large extent ineffective. At the present time, it would seem that the EDTA-induced lesions on the smooth surfaces of rat molars had a high similarity to true carious lesions. One of the perplexing facets of our studies has been the lack of any striking influence of the EDTA supplements on occlusal caries. Martin et al. ('54) have postulated that chelation may be an important part of the decalcification of tooth substance. If this postulate has any validity, it would be expected to hold for occlusal caries as well as for smooth-surface caries. Further definition of this comparison will require more exacting experiments than have been conducted.

#### SUMMARY

1. Ethylene diamine tetraacetic acid (EDTA), when added to a high sucrose cariogenic diet, produced lesions on the smooth surfaces of the molar teeth of white rats, which grossly resembled simple decalcification. The incidence and severity of these lesions increased in proportion to the concentration of EDTA in the diet.

2. EDTA failed to produce this type of lesion when added to a carbohydrate-free, high-protein, high-fat, non-cariogenic diet.

Of all the factors tested, the most important seemed to be the method of substitution of the test products in the basal ration. Thus in every instance, higher assay values were obtained both for dried skim milk and for crude casein when the assay rations were adjusted for the components of the test product than when substitution was made for dextrin or for corn meal and soybean meal. With the animal protein assay ration, such adjustment was easily accomplished with the test ration, since the basal ration already contained lactose and casein. With the vegetable protein ration, it was necessary to adjust the basal ration itself (including, obviously, the rations containing the B<sub>12</sub> standard) rather than the test ration. In the case of crude casein, adjustment was made in one case (assay no. 18) by including an equal amount of B<sub>12</sub>-deficient casein in the basal ration and in the other case (assay no. 19) by equating the protein level of both basal and test rations by adjustment of the proportions of corn and soybean meal. The lowered assay values observed when such adjustments were not made can no doubt be attributed primarily, if not altogether, to the depressing effect of lactose or an increased protein level or both upon growth of rats fed B<sub>12</sub>-deficient diets (Hartman et al., '49a, b). It may be pointed out that in the hyperthyroid rat assay method whereby certain literature values for the B<sub>12</sub>-content of milk products were obtained (Lewis et al., '49), substitution of the test product was made by a method not very different from the one used here that gave low assay values.

Thus in these comparative assays, essentially the same values were obtained with crude casein in all cases when the rations were adjusted so as to all have the same protein level. With dried skim milk, on the other hand, assay values were affected not only by the method of substitution of the milk but also by the type of ration used, by the method of depletion of the young and by the amount, although not by the presence, of iodinated casein in the ration.

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glucuronide, kynurenic acid (Brown and Price, '56) and *N*-methyl-2-pyridone-5-carboxamide (pyridone) (Price, '54) has now made it possible to obtain quantitative data concerning several other major metabolites of tryptophan. The use of these procedures (Brown and Price, '56) indicated that administration of a supplemental dose of *L*-tryptophan to man was followed by an increased urinary excretion of several of these metabolites.

In an effort to learn more about the metabolic fate of dietary tryptophan, studies have now been done on human subjects ingesting a constant amount of tryptophan and nicotinic acid. The use of a constant diet has also made it possible to determine the daily variation in excretion of the major known metabolites of tryptophan and to determine the relative importance of nicotinic acid and these other metabolites. In addition, the effect of a single oral dose of 2.0 gm of *L*-tryptophan on the excretion of these metabolites was determined. These results have been compared with similar studies on subjects ingesting self-selected diets (Brown and Price, '56).

#### EXPERIMENTAL

*Subjects.* The 4 subjects used in these experiments were male laboratory personnel ranging in age from 25 to 35 years. One subject was Japanese, one was from India, while the others were white Americans. None of the subjects had any history of neoplastic, renal, gastrointestinal or metabolic disease, and all were in apparent good health. During the studies each subject engaged in his usual activities. One subject was unable to complete the last two days of the experiment because the amount of food was excessive for him.

*Diet.* The diet consisted of natural foods listed in table 1. The food was prepared in the special diet kitchen by the hospital dietitians. All the food was taken from the same individual sources, with the exception of milk, lettuce, butter and eggs. Thus the canned goods were from the same case lot, the meat was from one beef round, the bread was from

# NUTRITIONAL STUDIES WITH THE GUINEA PIG

## IV. FOLIC ACID

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Folic acid was shown to be a dietary essential for the guinea pig by Woolley and Sprince ('45), a finding which has since been confirmed by other workers (Mannerling, '49; Woodruff, Clark and Bridgeforth, '53; Reid, '53). A marked folic acid deficiency, indicated not only by growth failure and short survival time but also by profoundly changed values for erythrocytes, hemoglobin, hematocrit, and total leucocytes, was produced (Reid, '54) in very young animals without the use of an antimetabolite. Presumably the animals employed in the studies of Woolley and Sprince were also quite young. Woodruff, Clark and Bridgeforth ('53), using 8-week-old animals weighing 250 to 300 gm, produced a deficiency as evidenced by a disturbed blood picture either by the omission of para-aminobenzoic acid (PABA) from a purified diet deficient in folic acid or by the inclusion of 1% sulfasuxidine with or without PABA. Wichmann, Salminen and Roine ('54), using a diet containing PABA, concluded that a dietary supply of folic acid was not essential because intestinal synthesis was thought to be sufficient to satisfy the folic acid requirement. Their animals had an average weight of 198 gm at the start and presumably were about three weeks old. The variations in the findings of these investigators have probably resulted from both the differences in age at which

mined by the method of Rosen et al. ('51), and *N*-methyl-2-pyridone-5-carboxamide (pyridone) which was determined according to Price ('54) as modified slightly by Walters et al. ('55).

## RESULTS

The results are given in table 2. The average pyridone excretion for the 4 subjects increased somewhat the second day on the diet, which suggests that more nicotinic acid was being ingested. From the second to the 6th days on the constant diet the average pyridone excretion was quite constant, ranging from 101 to 110 micromoles per day. The 4 subjects stabilized at different basal levels of excretion; for example, the average basal excretions of pyridone with standard deviations for each of the 4 subjects were  $80 \pm 10$ ,  $106 \pm 8$ ,  $112 \pm 11$  and  $127 \pm 5$  micromoles per day. For this reason the standard deviations shown in table 2 were much larger than the standard deviations of each individual's average basal excretion. After ingestion of the single supplement of 9.8 millimoles (2.0 gm) of L-tryptophan the pyridone excretion of each subject increased, and remained elevated for three days. One subject had an unexplained increase in pyridone excretion on the last two days of the experiment.

Kynurenic acid excretion varied little from day to day. The individual average basal excretions of this metabolite by the 4 subjects were  $13 \pm 1$ ,  $13 \pm 2$ ,  $18 \pm 1$ , and  $19 \pm 1$  micromoles per day.

The daily excretion of xanthurenic acid appeared to be quite variable on the basal diet, and the average values for the 4 subjects were  $27 \pm 3$ ,  $32 \pm 7$ ,  $38 \pm 9$ , and  $51 \pm 5$  micromoles per day. However, with low levels of xanthurenic acid this method of analysis gives somewhat variable results (Rosen et al., '51). There did appear to be a slight increase in xanthurenic acid excretion following the supplementation with tryptophan.

In agreement with the results of Brown and Price ('56) aromatic amine "Fraction A" was not affected by ingestion

before the 5th day on the diets. Between the 5th and 21st day an occasional animal (an average of two out of 100) succumbed for reasons other than the type of diet (e.g., broken leg, protruding intestine). This accounts for some of the groups reported in the tables having less than 6 or 8 animals.

For the collection of blood samples a modification of the method of Vallejo-Freire ('51) was used. A foot was held in warm water (45 to 47°C.) for 20 seconds to dilate the vessels and promote the blood flow after which a cut was made in the soft tissue near the insertion of the nail. Bleeding was stopped by tying off the toe above the cut.

## RESULTS

### *Growth and survival with different dietary levels of folic acid*

The average weights<sup>3</sup> at successive periods and the number of survivors, with levels of folic acid ranging from none to 2000 mg/kg of diet, are shown in table 1. With no folic acid in the diet there were no survivors in some experiments, whereas in others there were one or two at the end of the 6-week experimental period. With 1 mg of the vitamin per kilogram of diet, one-fourth of the animals survived. With 2 or 3 mg, survival was much improved but maximum growth was not attained. With 6 mg of folic acid growth was maximal and all of the animals survived. Growth at the 10 and 15 mg levels was not greater than at 6 mg. One animal in the 15 mg series died after a short illness, apparently of pneumonia. Since the tests with the 2000 mg level were made with the Beltsville strain of guinea pig, a somewhat smaller type than the Hartley strain, the growth rate of this group is not directly comparable with that of the Hartley strain. However, the average weights of the Beltsville group at the 2000 mg level slightly exceeded, though not significantly so, those of the

<sup>3</sup> Since the variations in weight from test to test were no larger than the variations among animals on the same diet on the same test the results from all tests on the same diet were pooled.

of single doses of L-tryptophan. The nature of the diazotizable aromatic amine in this fraction is unknown (Brown and Price, '56).

The average daily basal excretion of anthranilic acid glucuronide was  $3.0 \pm 0.6$ ,  $3.3 \pm 0.1$ ,  $5.2 \pm 0.7$ , and  $5.3 \pm 0.4$  micromoles; of o-aminohippuric acid  $17 \pm 1$ ,  $26 \pm 4$ ,  $31 \pm 2$ , and  $31 \pm 2$  micromoles; of N<sup>a</sup>-acetylkynurenine  $12 \pm 1$ ,  $12 \pm$

TABLE 3

*A comparison of the urinary excretion of tryptophan metabolites by subjects on the constant diet and on self-selected diet*<sup>1,2</sup>

METABOLITE	AVERAGE MICROMOLES EXCRETED PER SUBJECT PER DAY		AVERAGE MICROMOLE INCREASE IN EXCRE- TION AFTER 0.8 MILLI- MOLE DOSE OF L-TRYPTOPHAN	
	Constant diet	Self- selected diet	Constant diet	Self- selected diet
Pyridone	106	136	76	103
Kynurenic acid	16	16	51	51
Xanthurenic acid	37	75	13	11
Anthranilic acid glucuronide	4	6	2	3
o-Aminohippuric acid	26	27	31	23
Acetylkynurenine	15	9	4	4
Kynurenine	13	16	30	18
Total	217	285	207	213

<sup>1</sup> Brown and Price ('56).

<sup>2</sup> The average basal excretion of the metabolites includes days two to 6 on the constant diet and two days before tryptophan supplementation for the subjects on self-selected diets. The increased excretion of pyridone was calculated for a 4- or two-day period after administration of the supplemental tryptophan on the constant and self-selected diets, respectively. The increased excretion of the other metabolites included only the first 24 hours in both experiments.

2,  $17 \pm 3$ , and  $17 \pm 3$  micromoles; and of kynurenine  $9 \pm 1$ ,  $17 \pm 2$ ,  $16 \pm 3$ , and  $17 \pm 2$  micromoles for each of the 4 subjects. There was a slight increase in the excretion of each of these aromatic amines following the administration of the single dose of tryptophan, except that one subject failed to show an increased excretion of acetylkynurenine. This subject excreted more kynurenine than any of the other subjects, and may not have been as efficient in acetylation of this metabolite.

bone marrow was detected in histological studies by Dr. G. L. Fite.<sup>4</sup> The most outstanding signs of the deficiency were found to be in the blood picture as described in the following section.

*Blood studies of animals maintained on different levels of folic acid*

The results of these investigations are summarized in table 2. Insofar as was possible the blood studies were made near the end of the 6th week of the dietary regime. However, because of the short survival time of the unsupplemented controls and of the group receiving 1 mg of folic acid per kilogram of diet, blood studies of these animals had to be made considerably earlier. Since there were only 4 survivors in the no folic acid group, the values here shown may not be representative of the average picture. Other results for this group are shown later. The values found for the two groups (receiving no folic acid or 1 mg/kg) suggest a very poor condition with respect to both the red and white cells. With 2 mg of folic acid, the hematocrit was much improved and the leucocyte count was twice that found at the 1 mg level. With 3 mg/kg the values for the hematocrit, hemoglobin and erythrocyte counts appeared to be close to normal but the leucocyte number was not more than half that found at the higher levels of folic acid. The 6 mg level appeared to be adequate for producing normal blood values. No significant differences were found in either the red or white cell picture at folic acid levels of 6, 10, 15 and 40 mg.

*Rate of development of folic acid deficiency symptoms in the blood*

To obtain better information as to the rate of onset of the deficiency symptoms blood studies were made after varying periods of time on groups (6 to 8 animals) receiving no folic acid or 1, 2, 3 and 6 mg/kg of diet. The first determinations

<sup>4</sup> Laboratory of Pathology and Histochemistry, National Institutes of Health.

cient to account for about 85% of the estimated dietary niacin. On the basis of the studies by Walters et al. ('55) one might predict a conversion rate of this magnitude. Frazier, Prather and Hoene ('55) studied niacin metabolism in human subjects on a diet similar in niacin and tryptophan content to the present diet, and found that considerable amounts of *N*-methyl-nicotinamide were excreted. If the present subjects excreted similar amounts of this metabolite, over 100% of the dietary niacin would have been accounted for. This suggests that some tryptophan may be converted to niacin on a diet such as that used in the present studies. The urinary excretion of appreciable amounts of a number of the apparent by-products of the conversion of tryptophan to nicotinic acid also suggests that the subjects were using this metabolic pathway to some extent. The extent of the conversion of tryptophan to niacin on a natural diet presumably adequate in each might be difficult to obtain without the use of isotopes.

The pyridone excretion by the subjects studied by Frazier et al. ('55) would account for less of the dietary niacin than that which was accounted for in the present experiments. On a diet containing 16.3 mg of nicotinic acid and 1.12 gm of tryptophan the average pyridone excretion of their subjects was only 9.59 mg, which was enough to account for only about 48% of the dietary vitamin. However, Frazier et al. ('55) used a diet which was otherwise different from that used here. They also used female subjects and a different method for the determination of the pyridone.

From the data in table 3 it would appear that the pyridone was the chief urinary metabolite of tryptophan when a single supplemental dose of 2.0 gm of the amino acid was administered. From the increased urinary excretion of the other tryptophan metabolites, however, it would appear that considerable tryptophan was lost in side reactions along this pathway. Kynurenic acid was of most quantitative significance in this respect, followed closely by *o*-aminohippuric acid and kynurenine. That these other metabolites of tryptophan are by-products of the pathway leading to niacin

were made on the no folic acid, 1, and 2 mg groups after approximately two weeks on the diets and on the 3 and 6 mg groups after three weeks. The results (table 3) show little difference between the groups in the blood picture at this time but by the end of the third week the blood condition of the no folic acid group had deteriorated markedly, so much so that further tests were not possible. By the end of the

TABLE 3

*Changes in weight and in blood picture in relation to time and the folic acid content of the diet (6-8 animals per group)*

		FOLIC ACID (mg/kg)				
		0	1	2	3	6
Days on diet	(a)	13	14-17	14-17	21-24	21-24
	(b)	21	27-30	27-30	35-38	35-38
	(c)			42	49	49
Weight, gm	(a) <sup>1</sup>	146	156	159	207	219
	(b)	174	220	258	295	304
	(c)			339 <sup>2</sup>	376	386
Hematocrit, %	(a)	41.9	42.3	41.3	42.5	45.4
	(b)	35.7	30.4	35.9	41.1	44.9
	(c)			39.3	43.1	44.5
Hemoglobin, gm/100 ml	(a)	14.37	13.95	13.63	14.43	14.50
	(b)	13.98	10.18	11.76	13.15	14.52
	(c)			12.99	13.77	14.16
Erythrocytes, cells $\times 10^6/\text{mm}^3$	(a)	6.27	6.00	5.55	6.25	6.13
	(b)	5.56	4.07	4.73	5.74	5.70
	(c)			5.66	6.14	5.57
Mean corpuscular volume, $\mu^3$	(a)	66.9	70.3	74.0	68.2	75.4
	(b)	65.1	77.5	75.6	72.1	79.4
	(c)			69.7	70.1	77.5
Total leucocytes, cells/mm <sup>3</sup>	(a)	3225	3877	4410	3475	3508
	(b)	1722	2015	2190	2433	4400
	(c)			2800	2840	4350
Granulocytes, cells/mm <sup>3</sup>	(a)	1587	1627	1830	1375	1117
	(b)	364	641	530	642	1717
	(c)			1010	940	1817

<sup>1</sup> Weight after days on diet shown in (a) above, etc.

<sup>2</sup> Increase in weight and improvement in blood picture resulted from coprophagy.



L-tryptophan was given as a single oral dose and the excretion of the metabolites was determined for 6 more days.

On the constant diet about 16 mg of pyridone were excreted per day. The other metabolites of tryptophan accounted for 2.5% of the amino acid.

Following the ingestion of a single 2.0 gm supplement of tryptophan the pyridone excretion increased to an extent which would account for about 0.8% of the dose. The increase in the excretion of the other metabolites accounted for 1.3% of the supplemental tryptophan. Kynurenic acid, *o*-aminohippuric acid, and kynurenine were important urinary metabolites of the oral supplement of amino acid. Xanthurenic acid, acetylkynurenine, and anthranilic acid glucuronide were minor metabolites by comparison.

A comparison of these results with data of a similar nature obtained with human subjects on a self-selected diet suggested that a constant diet was not necessary for quantitative studies on the metabolism of supplemental doses of tryptophan, unless the conversion to nicotinic acid and its metabolites was of particular interest.

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the diets of one set of each of these three groups 100 mg of PABA were added. With no folic acid in the diet the addition of PABA was beneficial both as to growth and survival as is shown in table 4. With 2 mg of folic acid per kilogram, the addition of PABA appeared to have a slight beneficial effect on growth up to the 4th week but thereafter the difference faded out. With the 10 mg level of folic acid no beneficial effect of the PABA was seen during any part of the experimental period.

*Effect of variations in the ascorbic acid level  
in influencing the response to different  
levels of folic acid*

*Growth and survival.* Four experiments were conducted with ascorbic acid levels ranging from none to 5 gm/kg of diet with folic acid levels varying from none to 10 mg/kg. The general plan of the experiments and the results with respect to growth and survival are shown in table 5. Deficiency of both vitamins had a greater retarding effect on growth than the deficiency of either vitamin alone. The double deficiency shortened the average survival time to 19.5 days as compared to 24 days for a deficiency of folic acid alone and 26 days for a deficiency of ascorbic acid alone. In agreement with the results of Silverman and Mackler ('51), folic acid did not have an antiscorbutic effect. With a diet lacking folic acid, the addition of as much as 2 gm of ascorbic acid per kilogram of diet resulted in no apparent improvement over that obtained with only 50 mg, an amount known to be sufficient to prevent macroscopic symptoms of scurvy but insufficient to permit the development of a normal tooth structure (Reid, '54). With 1 mg of dietary folic acid the survival time, with only 50 mg of ascorbic acid, was slightly less than with higher ascorbic acid levels. At this folic acid level no differences in growth and survival were observed in the 1, 2, 2.5 and 5 gm levels of ascorbic acid. With 6 and 10 mg/kg of folic acid and 1 and 5 gm levels of ascorbic acid the growth



difference, if any, between the counts at the two ascorbic acid levels. Since the number of determinations under any one age and set of conditions was small (2 to 5), the leucocyte values for the entire period were averaged.

TABLE 6

*Total number of leucocytes as affected by the amounts of folic acid and ascorbic acid supplied*  
(8 animals per group)

FOLIC ACID AND ASCORBIC ACID SUPPLIED	LEUCOCYTES (cells/mm <sup>3</sup> )					
	Days on diet					Average
	28	35	42	51-61	66-68	
Folic acid, 2 mg/kg and Ascorbic acid						
100 mg/kg	2875	2725	3400	3025	3125	3030
200 mg/kg	3675	5025	4300	3700	4900	4320
Folic acid, 4 mg/kg and Ascorbic acid						
100 mg/kg	4275	4475	4175	4725	4000	4330
200 mg/kg	4525	3725	5125	4533	5175	4677
Folic acid, 6 mg/kg and Ascorbic acid						
100 mg/kg	4500	5100	5400	4812	4600	4882
200 mg/kg	5025	6900	4337	5200	5725	5437

## DISCUSSION

The young guinea pig quickly develops folic acid deficiency by the mere exclusion of the vitamin from the diet. The possibility of avoiding the use of antimetabolites, with the danger of resultant complications, should make the guinea pig a useful animal for studying the physiological action of this vitamin.

The young guinea pig appears to have a higher requirement for folic acid than any other animal thus far studied. During the present investigations which have extended over a period

Research Council, which for more than 25 years has been engaged in research on the physical, physiological and psychological growth and development of a group of children in the Denver area. These children, who come from "upper middle class" families, have been voluntarily enrolled in the study by their parents and are under the care of pediatricians in private practice. Since the purpose of this organization is research rather than therapy, no attempt has been made by the Council staff to influence the food intake of the children.

The nutrition data are based on a series of histories obtained by interview during home visits, with 4 consecutive 24-hour intakes recorded by the nutritionist and the mother. Histories are taken at monthly intervals during the first 6 months of life and thereafter at intervals of three months. Nutrients are calculated from food value tables (Bowes and Church, '56; U. S. Department of Agriculture, '48 and '50).

The data in this paper represent 1008 histories on 64 children (30 boys and 34 girls) who now range in age from 6 months to 12 years. Only the first 5 years of life are included. Intakes of breast-fed infants during the period of such feeding and two single histories on older children who had illnesses of sufficient severity to decrease markedly their food consumption during an entire three-month period have been excluded; all other histories taken during this age span on these 64 children have been included.

#### RESULTS AND DISCUSSION

Because the intakes of this group show a skewed rather than a normal distribution, the data are presented as 25th, 50th and 75th percentiles rather than as means and standard deviations. The percentile curves have been smoothed visually. The highest and lowest intakes observed are also indicated to give a picture of the very wide range in intake among children who are healthy and whose growth rates are satisfactory.

The dietary intake of vitamin A is presented as total intake, with separate figures for the contribution of animal and plant

obtained was 6 mg/kg of diet. However, the minimum requirement may be somewhat less than this amount since in the early phases of these investigations no levels were run between 3 and 6 mg. This high requirement of folic acid for the production of the normal leucocyte picture is similar to the findings of Campbell, Brown and Bennett ('44) for the chick. However, they found a 10-fold higher requirement for normal leucocyte production than for growth. The difference between the folic acid requirement for growth and for leucocyte production in the guinea pig is much less but may be as much as two-fold. More study is necessary before a more exact quantitative relationship can be stated. Although the growth rate was close to maximum at the 3 mg level, there usually were some fatalities in these groups. Presumably the leucocyte number and possibly antibody production also were not sufficient to give adequate protection against spontaneous infection. That folic acid plays an important role in protecting the animal against bacterial infection has been well demonstrated (Little, Oleson and Roesch, '50; Ludovici and Axelrod, '51; Wertman, Crisley and Sarandria, '52; Asenjo, '54).

The only other study on the relation of folic acid to growth and the maintenance of a normal blood picture in the guinea pig is that of Woodruff et al. ('53) in which they found that 3 mg/kg of diet were adequate to prevent blood changes for at least 8 weeks. However, it did not permit a daily growth rate equal to that of a commercial stock diet. Two factors contribute to the apparent discrepancy between their results and ours; namely, they started their tests with much heavier and older animals and their basal diet contained PABA. An additional third factor may be that of a difference in genetic strain of the experimental animals. In comparison with the blood values found in our animals, theirs had lower erythrocyte counts, slightly lower hemoglobin values, higher hematocrits and greater volume of the red cells.

In common with other animals (Bethell, '54), the guinea pig appeared to have a high tolerance for folic acid (2000

TABLE 1  
Vitamin A intake of children from birth to 5 years of age

AGE	NO. OF CASES	TOTAL DIET AND CONCENTRATE, I.U.					TOTAL DIET ONLY, I.U.				
		Percentile					Percentile				
		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
<i>years months</i>											
0-0 to 0-1	32	700	1000	1600	3400	10,400	200	800	950	1100	2400
0-1 to 0-2	38	1500	3500	4700	6700	12,350	900	1150	1400	1500	2100
0-2 to 0-3	39	1700	4300	6000	8500	15,715	800	1400	1600	1900	3600
0-3 to 0-4	42	2650	5000	7000	9600	14,720	800	1600	2000	2700	3900
0-4 to 0-5	44	3365	5800	7900	10,600	15,900	600	2100	3000	3700	6100
0-5 to 0-6	46	3230	6300	8600	11,800	16,300	550	2800	3700	5200	7500
0-6 to 0-9	48	5360	7300	9800	13,300	19,035	3000	4000	5100	6400	11,700
0-9 to 1-0	50	4000	7300	10,100	12,600	22,515	2800	4750	5900	7300	11,900
1-0 to 1-3	51	3600	6800	9000	11,000	21,900	1700	4600	6000	7500	11,200
1-3 to 1-6	51	3200	5700	7900	10,000	24,500	2000	3500	5100	6800	10,600
1-6 to 1-9	49	2285	5000	6900	9500	23,500	1600	2900	4200	5900	11,000
1-9 to 2-0	49	1900	4500	6200	9000	20,300	1100	2700	3800	5400	10,400
2-0 to 2-3	46	2500	4100	5800	8700	15,120	1400	2500	3500	5100	13,200
2-3 to 2-6	45	1900	3900	5500	8400	16,600	1500	2400	3400	4900	8300
2-6 to 2-9	43	1900	3600	5400	8300	16,100	1600	2400	3400	4800	8000
2-9 to 3-0	43	1800	3500	5300	8200	11,600	1100	2400	3400	4700	8200
3-0 to 3-3	41	1300	3400	5400	8200	14,100	1300	2400	3500	4650	11,100
3-3 to 3-6	38	1500	3500	5600	8200	16,450	1500	2500	3700	4650	7600
3-6 to 3-9	38	1300	3700	5750	8400	17,550	1100	2600	3800	4700	8900
3-9 to 4-0	34	2500	3900	5900	8600	14,860	1100	2750	3900	4800	6500
4-0 to 4-3	35	1900	4100	6000	8700	24,455	1900	2900	4000	4900	6800
4-3 to 4-6	35	1800	4300	6100	8800	24,455	1500	3000	4100	5050	6200
4-6 to 4-9	35	2100	4400	6200	8900	17,100	1100	3100	4200	5150	6600
4-9 to 5-0	34	2400	4500	6300	8900	17,100	1500	3200	4250	5300	7100

Hamilton and Stewart, '53) in not requiring an abundance of ascorbic acid to effect the conversion.

#### SUMMARY

Folic acid deficiency can be produced in the young guinea pig by the mere exclusion of the vitamin from the diet.

Folic acid deficiency is characterized by retardation of growth, gradual loss of appetite and activity, weakness, tendency to diarrhea, profuse salivation in the late stages, tendency to fatty infiltration of the liver and adrenal hemorrhages, an aplastic condition of the bone marrow, leucopenia and anemia.

The young guinea pig has an unusually high requirement for folic acid. From 3 to 6 mg of the vitamin per kilogram of diet is the minimum requirement for growth and the production of the normal red blood cell picture. The requirement is higher (6 mg or more) for producing and maintaining a normal leucocyte count. In the presence of 2 mg or less per kilogram of dietary folic acid, 200 mg of ascorbic acid per kilogram stimulates the production of leucocytes. With respect to growth and survival, ascorbic acid does not spare folic acid.

Added PABA is not a dietary essential for the guinea pig but it has an important supplementary value if the dietary supply of folic acid is inadequate.

At the age of 6 weeks the Hartley strain guinea pig reared on a complete diet had the following average blood values: hematocrit, 42.4; hemoglobin, 14.3; erythrocytes, 5.65; mean corpuscular volume, 75; total leucocytes, 4800; granulocytes, 1400.

#### ACKNOWLEDGMENTS

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In figure 1, in which the medians of these vitamin A sources are shown for comparison with each other, it may be seen that the curve representing total dietary vitamin A reflects the marked peak in plant sources. In considering the proportion of total dietary vitamin A which is supplied by plant sources, one finds that the level, which is at zero during the first two

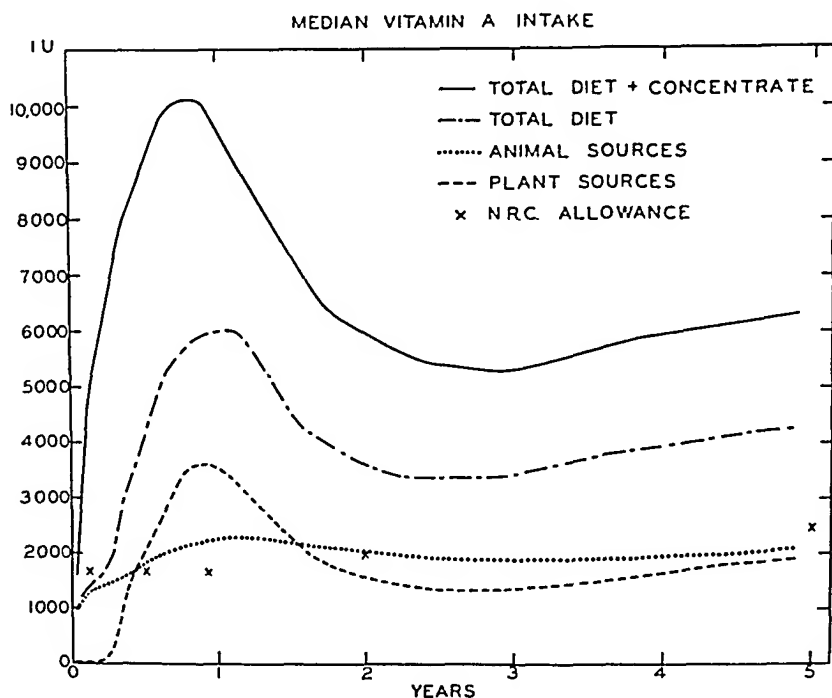


Fig. 1 Median intake of vitamin A from various sources contrasted with the Recommended Dietary Allowance in the first 5 years of life.

months, rises rapidly to 60% by one year, decreases to 40% by two and one-half to three years, then rises to nearly 50% by 5 years. However, at each age level there is a very wide range from minimum to maximum; for example, at two and one-half years the range is from 10 to 80% and at 5 years from 20 to 68%. Even though some additional carotene is supplied by animal foods, it seems unlikely that as much as two-thirds of the vitamin A in the diets of these children is

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of intake, they are typical of the group in that there is a tendency for each individual child to maintain a fairly constant position in the group. The only marked sex differences in this age span occur between 6 months and three years. The boys show a higher intake of plant sources at the end of the first year and a greater drop in both animal and plant sources during the early preschool period than do the girls.

Concentrates of vitamin A, with at least one other vitamin, are given to these children approximately two-thirds of the time during the first 5 years of life. The average child in our series who has reached his 5th birthday has received a vitamin concentrate 64% of the time, although the range is from 17 to 98%. It should be noted, however, that only in a few cases is the concentrate given daily; the average frequency is 4 to 5 times weekly. The frequency has been determined at each history so that the proper adjustment of actual intake could be made. When the vitamin A supplied by concentrates is added to the dietary intake, the total is far in excess of the Recommended Allowance. At one year, for example, the median total intake is 6 times greater than the Allowance, and by 5 years it is still two and one-half times greater. Of more concern is the fact that approximately 10% of these children have received intakes 10 to 15 times higher than the Allowance throughout the first year. Although no symptoms of hypervitaminosis A have been observed, the intake seems far in excess of need. Indeed, dietary sources alone supply adequate amounts of vitamin A for the majority of these children. In the past few years there has been a tendency toward use of vitamin preparations lower in vitamin A content than previously. Between 1946 and 1949, 62% of these children were given concentrates containing more than 10,000 I.U. of vitamin A per 10-drop dose, with additional vitamin D; since 1950 this has dropped to 8%, with the remainder of the children receiving concentrates with 5000 I.U. of vitamin A per 10 drops, most commonly multi-vitamin preparations.

Intake of vitamin D is presented in table 3. The level is high during the first year, due both to concentrates and to the

# PHYSIOLOGICAL AVAILABILITY OF THIAMINE FROM POTATOES AND FROM BROWN RICE<sup>1</sup>

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## TWO FIGURES

The concentration of thiamine in a food does not necessarily indicate how well that food will serve as a dietary source of this essential nutrient. The physiological availability of the thiamine in a particular food may be influenced by factors such as other components of the diet, a change in the intestinal microflora or the absence of some factor needed for utilization of thiamine. Parsons et al. ('45) reported that live yeast cells ingested by human subjects competed with the host for dietary thiamine. Green and co-workers ('41) found that foxes died of thiamine deficiency when certain raw fish were included in an otherwise thiamine-adequate diet. Melnick et al. ('45) found that thiamine was destroyed in the intestinal tract when raw clams were eaten. Later work (Sealock and Davis, '49) showed that raw fish and clams contain the enzyme thiaminase which catalyzes the hydrolytic splitting of thiamine into the pyrimidine and thiamine moieties. Ensminger et al. ('45) found that pigs fed natural rations deposited thiamine in the tissues more efficiently than pigs on purified rations; they believed that certain factors present in the natural rations but not supplied in the purified rations were needed for optimal utilization and deposition of thiamine in the animal tissues.

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TABLE 4

*Ascorbic acid intake of children from birth to 5 years of age*

AGE	NO. OF CASES	DIET AND SUPPLEMENT, mg					DIET ONLY, mg					
		Percentile					Percentile					
		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest	
<i>Years months</i>												
0-0 to 0-1	32	0	0	0	16	86	0	0	0	0	0	2
0-1 to 0-2	38	0	12	20	32	71	0	0	0	0	4	19
0-2 to 0-3	39	4	19	26	44	88	0	0	0	0	7	32
0-3 to 0-4	42	0	22	31	50	97	0	0	0	2	12	29
0-4 to 0-5	44	4	26	35	55	97	0	3	5	8	20	46
0-5 to 0-6	46	6	29	39	60	99	0	5	8	26	56	56
0-6 to 0-9	48	6	33	46	66	101	4	9	19	36	71	71
0-9 to 1-0	50	6	38	53	72	141	3	15	31	49	91	91
1-0 to 1-3	51	11	42	58	77	123	5	22	40	58	102	102
1-3 to 1-6	51	6	45	62	81	131	6	27	46	64	105	105
1-6 to 1-9	49	6	47	65	84	131	6	31	49	69	111	111
1-9 to 2-0	49	13	48	66	87	164	13	33	51	72	114	114
2-0 to 2-3	46	12	49	67	89	149	12	34	52	73	149	149
2-3 to 2-6	45	25	49	68	91	159	11	35	53	74	159	159
2-6 to 2-9	43	11	50	69	93	205	11	36	54	75	205	205
2-9 to 3-0	43	19	50	70	95	196	15	37	54	76	196	196
3-0 to 3-3	41	12	51	71	97	118	12	38	55	77	84	84
3-3 to 3-6	38	22	52	72	99	130	13	39	55	78	95	95
3-6 to 3-9	38	15	52	73	101	135	5	41	56	80	130	130
3-9 to 4-0	34	32	53	74	103	153	15	42	56	82	105	105
4-0 to 4-3	35	14	54	75	105	164	12	44	57	83	126	126
4-3 to 4-6	35	22	54	75	107	141	21	45	58	84	141	141
4-6 to 4-9	35	23	55	75	109	189	17	46	59	85	189	189
4-9 to 5-0	34	15	55	75	110	167	15	47	60	85	167	167

The test foods replaced comparable foods in the control diet — potatoes replaced brown rice and whole wheat bread, lamb replaced meat loaf and peanut butter — so that approximately one-third of the day's thiamine intake came from the test food. The potato test diet contained 200 gm of baked potatoes and the lamb test diet 120 gm of roast lamb at each of two meals. The lamb-and-potato test diet contained 200 gm of potato and 120 gm of lamb at one meal. The control diet contained, by analysis, 0.88 mg of thiamine, the potato test diet, 1.04 mg, the lamb test diet, 0.79 mg, and the lamb-and-potato test diet, 0.85 mg. Each test diet was eaten for a two-day period. This short test period was used to give a measure of the availability of the thiamine in the test foods as they are used in an ordinary diet. The test-diet periods were preceded, separated and followed by two-day periods on the control diet. The series was concluded with a period in which unenriched white bread and white rice replaced the whole wheat bread and brown rice in the control diet, and pure thiamine was given to make the total thiamine intake equal to that on the control diet.

Complete 24-hour urine collections were made throughout the study. The collection bottles contained a preservative of acetic acid and ethyl alcohol; aliquots for assay were refrigerated under toluene. Thiamine determinations on the foods and the urine samples were done by a modification of the thiochrome procedure of Hennessy and Cerecedo ('39) using the Coleman photofluorometer.

### *Results*

The data for thiamine intake and excretion of each subject during the periods on the test diets and the control diet are given in table 1. The percentage excretion is shown graphically in figure 1.

For all subjects in series A, less thiamine was found in the 24-hour urine samples when potatoes were the important source of thiamine than when any of the other experimental

preschool period. There is, instead, a plateau followed by a slight rise. The intakes of individual children maintain a remarkable constancy of position in the group. For example, a child whose intake at two years is at the 25th percentile of the group tends to maintain a similar level through 5 years.

#### SUMMARY

Data have been presented from 1008 nutrition histories on 64 children in the first 5 years of life. Intakes of vitamins A and D and of ascorbic acid have been computed in terms of quartiles and of maximum and minimum levels observed. Some individual patterns of intake of vitamin A are shown.

The intake of animal sources of vitamin A shows little variation from age to age and a relatively small range at any age. The intake of plant sources, however, shows much more variation; there is a marked peak at the end of the first year, a decline in the second and third years, and a rise following three years of age. After the first 4 months, plant sources supply an average of 40 to 60% of total dietary vitamin A. After the first three months more than three-fourths of these children exceed the N. R. C. Recommended Allowance in vitamin A intake from diet alone; in addition, vitamin concentrates are given an average of 64% of the time.

The median vitamin D intake increases to a peak of 1000 I.U. daily at 4 to 6 months, then decreases to a level just below 400 I.U. daily by 5 years.

During the first 6 to 9 months most of the ascorbic acid intake is from ascorbic acid preparations rather than from diet; thereafter diet supplies an increasingly larger amount. After two years the Recommended Allowance is approximately at the 25th percentile level of observed intake from diet alone.

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not significantly different from that excreted during the corresponding periods on the control diet.

The first part of this study was repeated. The 4 subjects in series B also excreted less thiamine on the potato test-diet than on the control diet (an average of 19.5% for the control periods and 15.2% for the potato test-period). Statistical analysis of the data showed that these differences were highly significant.

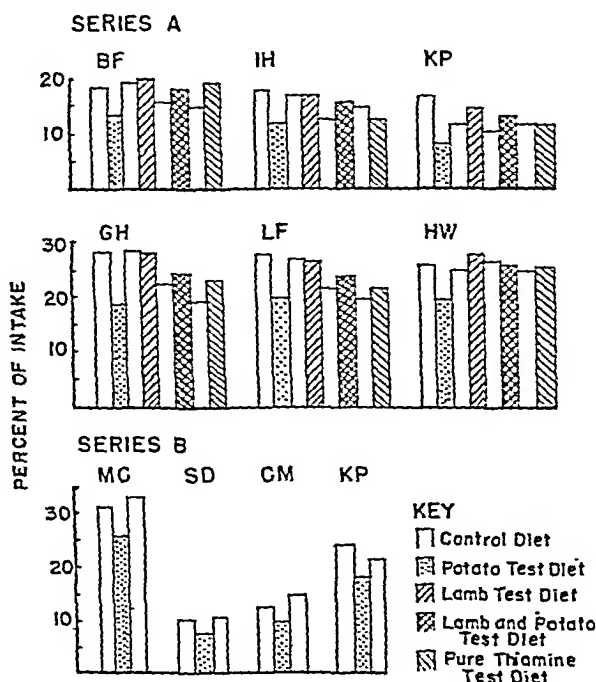


Fig. 1 Percentages of the thiamine intake excreted in the urine by human subjects on 5 test diets.

Because potatoes yield an alkaline residue, it was postulated that the potato test-diet might have changed the pH of the urine sufficiently to cause destruction of thiamine while the urine was in the bladder. Solutions of thiamine in  $\text{Na}_2\text{HPO}_4 - \text{KH}_2\text{PO}_4$  buffers at pH 6.79 and lower retained 96% of the thiamine after one hour at 37°C., but the solution at pH 6.83 retained only 80%. A urine sample with a pH of





The dry feed in the control diet had the following composition in pounds: barley, 30; oats, 30; wheat, 25; sun-cured alfalfa, meat meal and fish meal, 3.3 each; bone meal, 0.5; skimmilk powder, 50. For the dry feed in the test diets, the amounts of barley, oats and wheat were reduced by one-half, the alfalfa, meat meal and fish meal were increased to 5.0 lb. each and the bone meal and skimmilk powder remained the same. The control-diet dry feed, which all pigs received for an adjustment period of 7 days, was mixed with water in a 1:2 ratio. The test diets were fed in the ratio of one part of test-diet dry feed, two parts of cooked test-food (potatoes or brown rice) and three parts of water. One pound of the control-diet dry feed contained essentially the same concentration of nutrients as  $\frac{3}{4}$  lb. of the test-diet dry feed plus  $1\frac{1}{2}$  lb. of cooked test-food. The concentration of nutrients in the three diets was calculated to exceed the nutrient allowances for swine recommended by the National Research Council ('50). The potatoes and rice were cooked daily and refrigerated until used. Thiamine losses during 24-hour storage were negligible for the rice and did not exceed 10% for the potatoes.

The pigs were housed in the individual metabolism cages described by Lehrer and Wiese ('53). They were fed 4 times a day at 4-hour intervals; water was offered after each feeding. Special feeding pans, designed to minimize feed losses from spilling, consisted of loaf pans mounted in shallow drip pans on heavy boards. The dry feed, test food and water were thoroughly mixed in the pans, in amounts based on the quantity of food each pig had eaten at the previous meal. The pans were weighed before and after each feeding to determine food intake. The pigs were weighed weekly.

In the first group of pigs, 5 were 4-week-old Poland China litter-mates and two were  $5\frac{1}{2}$ -week-old Duroc litter-mates.\* The 7 pigs in group 2 were 4-week-old Durocs from a single litter.

\* A high mortality rate in the Poland China litter reserved for this study made the substitution of the two Durocs necessary.

known to produce a very high incidence of exudative diathesis (Scott et al., '55). The control group received this diet supplemented with a high level<sup>3</sup> of vitamin E.

Blood was obtained by cardiac puncture, 1 ml being removed into a hypodermic syringe which had been rinsed with a heparin sodium solution.<sup>4</sup> The blood was then transferred to a tube containing one drop of the same heparin solution and mixed gently by swirling. Initially, the exudate was collected (from the same chicks as the plasma) by killing and skinning the chicks and then aspirating the fluid into a heparin-rinsed syringe, the rest of the procedure being the same as for the blood. Later, in an effort to preserve the chicks for subsequent examination, the exudate was removed by perforating the skin with a 20-gauge hypodermic needle and expressing the fluid into the collecting tubes.

Total protein was determined by a semi-micro modification<sup>5</sup> of the biuret method of Gornall, Bardawill and David ('49). Hanging strip paper electrophoresis was employed using a "Durrum Type" cell<sup>6</sup> (Durrum, '50; Block et al., '55; Williams et al., '55). The buffer system was sodium barbital-barbituric acid of ionic strength 0.075 and pH 8.6. A sample of plasma or exudate (0.01 ml) was applied to each strip of Whatman no. 3MM filter paper. The separations were carried out for 16 hours at a constant current of 5 milliamperes for 8 strips in parallel, after which the strips were dried and stained with bromophenol blue as outlined in the technical manual pro-

<sup>3</sup> 20 mg of *d*- $\alpha$ -tocopheryl acetate per pound of diet.

<sup>4</sup> "Heparin Na for Injection." Lederle Laboratory Division of American Cyanamid Corp.

<sup>5</sup> The protein solution was mixed with enough water to make 1.5 ml and an equal volume of biuret reagent was added. After incubation at 38° for 15 minutes, the sample was read at 540 m $\mu$  in the Beckman Spectrophotometer against a reagent blank (1.5 ml of H<sub>2</sub>O, 1.5 ml of biuret reagent) which had also been incubated. One milligram of protein = D540 of 0.095. The upper limit of determination is 2.0 mg of protein, which produces a D540 of 0.190. From the unpublished notes of D G. Goldman appearing in the Methods Manual, Enzyme Institute, University of Wisconsin.

<sup>6</sup> "Model B" paper electrophoresis apparatus manufactured by Specialized Instruments Corp., Belmont, Calif.

TABLE 2  
*Weight gain of pigs on experimental diets*

GROUP	PIG NUMBER	SEX	DIET	WEIGHT		WEIGHT GAIN ON TEST DIET
				When received	Final	
				lb.	lb.	lb.
Group 1						
Poland China	4	F	Control	8	20	11
	1	M	Rice	12½	25½	12
	3	F	Rice	12	23	11
	5	M	Potato	12	24	11
	6	F	Potato	13	28	14
Duroc	2	M	Rice	27	45½	15½
	7	F	Potato	25	41½	14
Group 2						
Duroc	11	F	Control	17½	40½	24½
	12	M	Rice	14	41½	25½
	13	F	Rice	16½	38½	22½
	17	F	Rice	17	37½	19½
	14	F	Potato	14½	37	21
	15	M	Potato	18½	41½	21½
	16	F	Potato	19½	45½	23½

MUSCLE TISSUE  
 HAM, LOIN, SHOULDER

ORGANS  
 HEART, KIDNEY, LIVER

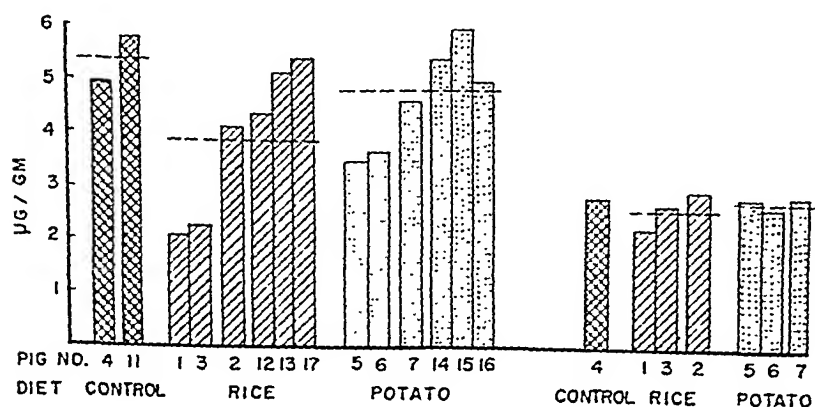


Fig. 2 Average concentration of thiamine in the muscle tissues and the organs of weanling pigs on three experimental diets.

vided with the apparatus. After color equilibrium had been reached, the stained strips were scanned with a servo-type integrating scanner<sup>7</sup> (Block et al., '55).

#### EXPERIMENTAL

*Part A. Electrophoretic patterns obtained from control and vitamin E-deficient chicks.* Moving-boundary electrophoresis of normal adult chicken plasma in veronal buffer has shown the usual 6 Tiselius components (Sanders et al., '44), whereas in borate buffer an  $\alpha$ 3-globulin fraction appears to be present (Brandt et al., '52). McKinley et al. ('54) by the use of paper electrophoresis have shown the presence of an  $\alpha$  3-globulin component in chickens aged 7 to 15 weeks. Our results with male chicks also showed an  $\alpha$  3-globulin component.

Only pooled samples (from 4 or more chicks) both of plasmas and exudates were used. For the control chicks, patterns were obtained from the plasmas at the following ages: one day (prior to being placed on diet), 9 days, 14 days, 21 days, 28 days and 35 days (fig. 1).

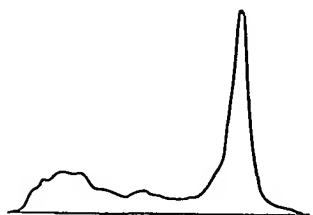


Fig. 3 Electrophoretic pattern of plasma from 28-day-old chicks receiving normal stock diet.

For the experimental chicks, patterns were obtained from the plasmas at the ages of 9, 14 and 21 days; exudate patterns, at 14 and 21 days (fig. 2).

Patterns were also obtained from the pooled plasma of 28-day-old chicks of the same sex and strain which had received a normal stock diet<sup>8</sup> (fig. 3).

*Part B. Administration of a high dose of d- $\alpha$ -tocopheryl acetate to chicks suffering from exudative diathesis.* Four 21-day-old birds suffering from severe exudates were given

<sup>7</sup> "Analytrol" scanner, manufactured by Specialized Instruments Corp., Belmont, Calif.

<sup>8</sup> Manufactured by Cooperative Grange League Federation Exchange, Inc., Ithaca, New York.

diet, but the concentration of thiamine in the muscle tissues of the pigs fed the potato diet was 64% higher. The Duroc in group 1 which was fed the rice diet received one-fourth more thiamine than the one fed the potato diet, but again the concentration of thiamine in the muscle tissues was higher for the pig fed the potato diet (9% higher).

In group 2, although there was considerable variation in intake and thiamine storage among individual animals receiving the same treatment, the average intake of thiamine was approximately the same for the pigs on both test diets, but the average thiamine concentration in the muscle tissues of the pigs fed the potato diet was 9% higher than that of the pigs fed the rice diet. Statistical analysis of the pooled data showed that the differences in thiamine concentration in the muscle tissues of the pigs on the two test diets were significant at the 1% level. There might have been greater differences in the thiamine storage on the two test diets if the experimental feeding period had been shorter. The large Durocs ate much more food than the Poland Chinas in group 1, and may have received sufficient thiamine from either diet so that the slower storage on the rice diet may have caught up with the more rapid storage of thiamine from potatoes.

The thiamine content of the urine samples collected in the latter study was low. The pigs fed the potato diet excreted an average of 3.1% of their intake (based on 12 observations) and the pigs fed the rice diet excreted an average of 4.2% (based on 17 observations). The pH of several samples of urine as voided was around 6.5, so the low thiamine content was probably not the result of destruction of the thiamine in the bladder due to alkalinity.

The data from these studies on young pigs suggest that the lower excretion of thiamine in the urine of the human subjects receiving important amounts of thiamine from potatoes may have been due to increased accumulation of the thiamine in the tissues. It is possible that both the deposition of dietary thiamine and the urinary excretion of the vitamin are in-

## DISCUSSION

*Part A.* The electrophoretic patterns of the plasmas from the control chicks all showed well-defined albumin peaks (fig. 1). The values for percentage of total protein in chicks receiving vitamin E, reported in table 1, were within the normal range reported by other workers (Brandt, et al., '51). The data presented in tables 1 and 2 show the percentage of total protein of the plasma to be lower for the vitamin E-deficient chicks than for the controls. A comparison of the electrophoretic patterns indicates that the exudates contained the same proteins as the blood plasma. At 14 days the vita-

TABLE 1

*Total protein and relative distribution of plasma proteins in chicks fed the experimental diet supplemented with vitamin E*

AGE WHEN BLOOD SAMPLE TAKEN	TOTAL PROTEIN	RELATIVE COMPOSITION						
		A/G <sup>1</sup>	a1/A	a2/A	a3/A	β/A	φ/A	γ/A
<i>days</i>	<i>%</i>							
1	3.56	0.39	0.26	0.59	0.24	1.07		0.38
9	3.79	0.57	0.26	0.23	0.17	0.69		0.40
14	2.46	0.47	0.30	0.37	0.17	0.70	0.20	0.40
21	3.56	0.70	0.27	0.27	0.16	0.39	0.19	0.29
28	3.41	0.43	0.37	0.26	0.20	1.03		0.43
35	3.45	0.43	0.35	0.38	0.24	0.62	0.31	0.41
28 COM <sup>2</sup>	3.16	1.12	0.20	0.14	0.11	0.28		0.15

<sup>1</sup> A = albumin, G = globulins

<sup>2</sup> Twenty-eight-day-old chicks on stock diet.

TABLE 2

*Total protein and relative distribution of plasma proteins in chicks fed the basal diet*

AGE WHEN BLOOD OR EXUDATES TAKEN	TOTAL PROTEIN	RELATIVE COMPOSITION						
		A/G	a1/A	a2/A	a3/A	β/A	φ/A	γ/A
<i>days</i>	<i>%</i>							
9 PL <sup>1</sup>	3.54	0.39	0.27	0.49	0.27	0.91		0.64
14 PL	2.30	0.17	1.10	0.80	0.50	2.30		0.90
14 EX	2.65	0.35	0.30	0.37	0.27	1.30		0.47
21 PL	2.44	0.20	1.00	0.95	0.50	1.10	0.65	0.95
21 EX	1.49	0.18	1.05	1.06	0.69	1.81		0.88

<sup>1</sup> PL = pooled plasmas, EX = pooled exudates.

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proved, although they still exhibited exudates. After three days, all signs of exudates were absent, and the physical condition of the chicks appeared to be comparable to that in the control group. The chicks remained normal for the remainder of the experiment even though blood samples were taken from them regularly.

Since, in the vitamin E-deficient chicks, the electrophoretic pattern of the exudate fluid was similar to that of the plasma, it appears that the results presented here agree with those of Dam and Glavind ('40) in showing that increased capillary permeability is an important factor in the production of exu-

TABLE 3  
*Data obtained from chicks before and after administration of 0.25 ml  
d- $\alpha$ -tocopheryl acetate*

CHICK 2171	TOTAL PROTEIN	RELATIVE COMPOSITION						
		A/G	$\alpha 1/\Lambda$	$\alpha 2/\Lambda$	$\alpha 3/\Lambda$	$\beta/\Lambda$	$\Phi/\Lambda$	$\gamma/\Lambda$
<i>days</i>	<i>%</i>							
0 <sup>1</sup>	1.98	0.10	2.25	1.63	1.00	4.13		17.5
2	2.40	0.14	1.14	1.14	0.43	2.22	1.00	1.14
3	2.86	0.31	0.74	0.48	0.24	1.28		0.62
5	3.18	0.39	0.39	0.33	0.24	0.33	0.61	0.42
7	3.01	0.39	0.42	0.49	0.31	0.44	0.50	0.36
9	3.16	0.39	0.56	0.40	0.20	0.56	0.48	0.36

<sup>1</sup> Prior to the administration of d- $\alpha$ -tocopheryl acetate.

dativc diathesis. However, reduced albumin concentration in the plasma because of failure of normal albumin synthesis during vitamin E deficiency may contribute to the edematous condition through lack of maintenance of normal osmotic relationships between the plasma and the tissue fluids. Dam ('44a), Hove ('46) and Moore ('49) have presented results with rats indicating that vitamin E is concerned in protein synthesis.

The failure of normal albumin synthesis during vitamin E deficiency in the chick is indicated by the low albumin concentrations in both the plasma and exudates, and by the fact that the plasma albumin level was rapidly restored upon administration of a single dose of d- $\alpha$ -tocopheryl acetate. Since

# REQUIREMENTS OF RATS FOR VITAMIN B<sub>12</sub> DURING GROWTH, REPRODUCTION AND LACTATION

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Large sectors of the human populations live on a more or less exclusively vegetarian diet. This fact induced us several years ago to initiate studies on the effect of such a type of feeding on experimental animals kept under similar conditions for many generations. Preliminary experiments showed the existence of some factor (Jaffé, '46), later identified as vitamin B<sub>12</sub> (Jaffé, '48) lacking in this kind of ration. Because vegetarian diets are low in this vitamin, it seemed interesting to conduct some long-range experiments on their effect on experimental animals. Moreover, an attempt has been made to determine the minimum vitamin B<sub>12</sub> requirements for growth, reproduction and lactation of the rat under conditions of uniform intake during more than one generation.

## EXPERIMENTAL

The animals were descendants of the "Sprague Dawley" strain. All the rats of the experimental series were from a stock kept on a soybean oil meal-corn ration since 1948, while a control group was always fed a commercial stock diet. The animals were housed in screen bottom cages in a room without air conditioning. Large litters were always reduced to 6 within 48 hours after birth, weaned at 21 or 28 days of age and kept together in a common cage to permit brother and sister mating for the following generation. In special cases,

Oral administration of 0.25 ml of pure *d*- $\alpha$ -tocopheryl acetate produced complete recovery of chicks suffering from exudative diathesis.

The possible role of vitamin E in capillary permeability and protein synthesis has been discussed.

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For the determinations of soluble, reduced nonprotein sulfhydryl compounds in the livers, a modification (Jaffé and Budowski, '54) of the Grunert and Phillips ('51) method for glutathione was used. Vitamin B<sub>12</sub> was determined with a modification of the USP method.

#### RESULTS

In table 1 data are presented on the reproduction of rats kept for 7 years on the soybean oil meal-corn ration. The enumeration of the generations starting with the 5th is only approximate as the groups were selected according to the date of the birth of their litters and the corresponding generation calculated only with the first and last animals of each group. Therefore, some overlapping may exist among the last 4 groups.

The litters in the first generation showed distinctly better performance than later ones with respect to the number of animals born in each litter, number of surviving animals, and weaning weights. This was to be expected, as the complete stock ration was fed during gestation. The performance in this group is virtually the same as for the animals kept on the stock ration for the whole lactation period. Starting with the second generation, a very considerable deterioration in the performance of the litters may be observed. The number of animals weaned per litter was only about half that in the first generation. As the number of young which died between the second day after birth and the weaning age was always small, it is clear that this is due mostly to the higher number of stillbirths or to deaths in the first two days of life. There was a tendency for all of the young in litters of certain mothers to die in contrast to a low death rate in the litters of others.

No difference between the first and succeeding generations could be observed with respect to the weight gain of the mothers during the 4 weeks of lactation or to the mean weight of the young at birth.



The results of various supplements of vitamin B<sub>12</sub> on reproduction and lactation performance are summarized in table 2. The performance of the animals on the basal diet was poor in comparison with the B<sub>12</sub>-supplemented groups with respect to the survival of litters, number of animals weaned per total litters born, weaning weights at 4 weeks and the age of the mothers at the birth of their first litters. The results of group 2 show that the addition of 3 µg of vitamin B<sub>12</sub> per kilogram of diet did not result in a similar weight gain of the young as

TABLE 3

*Post weaning growth of rats bred on different experimental diets*

DIET AND SUPPLEMENTS	NO. OF ANIMALS	SEX	WEIGHT AT 3 WEEKS	WEIGHT AT 7 WEEKS
			gm	gm
Basal	23	M	27.9 ± 0.9 <sup>1</sup>	119.2 ± 4.9 <sup>1</sup>
Basal	21	F	29.3 ± 2.1	109.2 ± 3.6
Basal + 3 µg/kg vit. B <sub>12</sub>	25	M	34.5 ± 1.1	154.5 ± 3.1
Basal + 3 µg/kg vit. B <sub>12</sub>	28	F	34.4 ± 1.0	123.5 ± 1.7
Basal + 5 µg/kg vit. B <sub>12</sub>	30	M	41.8 ± 0.6	170.2 ± 3.3
Basal + 5 µg/kg vit. B <sub>12</sub>	30	F	41.5 ± 0.7	137.0 ± 1.9
Stock	24	M	42.1 ± 0.9	163.6 ± 3.4
Stock	28	F	42.3 ± 1.8	134.4 ± 2.1

<sup>1</sup> Standard error of the mean.

that observed with larger supplements, although the performance was nearly the same in all other respects.

Supplements of 5 µg/kg of vitamin B<sub>12</sub>, and 30 µg/kg together with 0.2% of methionine gave identical results. A comparison between the group of rats fed the complete stock diet and the two latter supplemented groups shows that there were no significant differences in all those aspects studied in our experiments with the only exception of the litter size, the birth weight, and possibly the number of litters in which all animals died before weaning time. In experiments not in-

of a very slight change in the odor of the diets. It appeared on closer examination that change in odor, indicating the onset of rancidity, occurred more rapidly in diets made with dextrose than in those made with sucrose. As a result of these observations, the influence of the kind of carbohydrate in the diet upon development of rancidity was studied.

#### EXPERIMENTAL PROCEDURE

The diets used are shown in table 1. Small amounts of water soluble vitamins were added. In the first experiments the

TABLE 1  
*Composition of experimental diets*

CONSTITUENT	AMOUNT
	%
Sucrose <sup>1</sup> or Hydrate Dextrose <sup>2</sup>	64
Crude casein	20
Celluloflour	3
Salt mixture <sup>3</sup>	2.5
Corn oil	9
Cod liver oil	1
Choline chloride	0.2

<sup>1</sup> Revere Sugar Refining Co.

<sup>2</sup> Corn Products Refining Co.

<sup>3</sup> Jones and Foster ('42) mixture with calcium carbonate removed.

carbohydrates used were exactly as supplied by producers; in later experiments the carbohydrates were finely ground in a ball mill to insure uniformity in particle size. Other modifications will be indicated in the Results. The diets were thoroughly mixed and measured out in 5 gm aliquots in paper weighing cups. These cups were then placed in a 75°F. temperature-controlled room until analyzed. At appropriate times, fat from a sample was extracted with petroleum ether, the ether distilled off on a steam bath and the iodine number determined by the Wijs method (cited by A. O. A. C., '40). Duplicates were done until it was certain that the method was reproducible. The results of the iodine number assays are

mented and vitamin B<sub>12</sub>-supplemented groups; the stock animals however, had higher levels. Hemoglobin, hematocrit, and urea determinations as well as red and differential white blood cell counts were made on groups of adult male rats from groups 1, 3, and 5. All the values found were within the range accepted as normal. Vitamin B<sub>12</sub>-deficient animals had slightly higher values of blood urea than stock animals ( $0.27 \pm 0.017$  gm/l vs.  $0.24 \pm 0.016$  mg/l).

In table 5, the values for vitamin B<sub>12</sub> in livers and kidneys of adult male rats, kept on one of three different diets, are presented. It can be seen that the diet containing 5 µg/kg of vitamin B<sub>12</sub> did not cause as high tissue levels of this vitamin as the stock diets although the values were higher than they were with the basal ration.

#### DISCUSSION

In confirmation of Dryden et al. ('52), the data in tables 1 and 2 show that in mother rats fed a diet low in vitamin B<sub>12</sub> there is a tendency for the entire litters of certain mothers to die; this is in contrast to a relatively low mortality rate for the litters produced by other individuals. This suggests that by applying the principles of selective breeding it might be possible to develop a strain of rats that is relatively resistant to vitamin B<sub>12</sub> deficiency. We used mostly brother and sister matings in order to accentuate any such tendency.

Nevertheless, the differences observed between the different deficient groups in subsequent generations are small and of doubtful significance. The first deficient group (second to third generation) showed the poorest overall performance, but the observed differences are not impressive in view of the fluctuations between the following experimental groups. Between the third to 4th and 16th to 18th generations, no such selective trend was observable.

The lack of a significant difference between the first groups and the last, which were observed after 6 to 7 years of almost continued brother-sister breeding on the vitamin B<sub>12</sub>-low diet, is contrary to what we had expected to find. It may be related



hydrate effect persisted after grinding. The addition of an antioxidant<sup>2</sup> retarded the development of rancidity in both diets, but rancidity developed sooner in the presence of dextrose.

Data shown in table 4 are the result of diets made without the salt mix as well as diets made with anhydrous dextrose. Diets without the salt mix were much more stable than those with the salt. Anhydrous dextrose, however, was inferior to the other sugars.

In other tests not shown here, the previous work of White et al. ('53) was confirmed whereby the removal of choline

TABLE 4

*Effect of salt mixture and carbohydrate on development of rancidity*

DAY	SUCROSE		DEXTROSE <sup>1</sup>		ANHYDROUS DEXTROSE <sup>1</sup>	
	No salts	Plus salts	No salts	Plus salts	No salts	Plus salts
0	1.1	1.1	1.1	1.1	1.1	1.1
4	1.2	1.3	1.1	1.3	1.4	6.6
5	1.2	1.3	1.1	1.3	1.5	
7	1.2	1.3	1.1	6.6	6.5	13.2
8	1.2	1.3	1.1	11.3	10.6	13.2
11	1.2	3.4	1.3	14.4	14.0	15.0

<sup>1</sup> Results reported as milliliters of 0.1 N thiosulfate/0.5 gm of fat.

from the diets markedly improved stability. Although a few studies have been done, no effect of carbohydrate has been observed in the absence of choline.

#### SUMMARY

These studies have shown that the kind of carbohydrate used in a purified diet has an important effect upon the rate at which rancidity develops. Diets made with glucose were much more susceptible to auto-oxidation than those made with sucrose. The mechanism of the effect is unknown.

<sup>2</sup> A combination of butylated hydroxy-anisole, propyl gallate and citric acid. Supplied by Eastman Chemical Products Co., Kingsport, Tenn.

Although we were unable to detect the operation of a genetic selection of animals with better performance on a diet low in vitamin B<sub>12</sub>, the results presented in table 1 show that it is possible to breed rats for at least 18 and probably many more generations on a fortified soybean-corn ration not supplemented with vitamin B<sub>12</sub>.

The lack of vitamin B<sub>12</sub> in the diet caused a high mortality rate of the litters, a low birth weight and weaning weight, and the deficient females were older when they gave birth to their first litters. The first two deficiency symptoms were at least partially, and the latter two completely overcome by a supplement of only 5 µg/kg of vitamin B<sub>12</sub>, while the dose of 3 µg/kg was insufficient to secure normal weaning weight. As there was no difference observable between the effect of supplements of 5 or 30 µg/kg, the minimum dose must be somewhere between 3 and 5 µg/kg of vitamin B<sub>12</sub> in a soybean-corn diet under the present conditions.

The tendency of vitamin B<sub>12</sub> supplements to cause greater birth weights in rats, observed by other authors (Daniel et al., '53; Dryden et al., '51) can also be detected in our results, although the young born of mothers fed the stock ration were still heavier at birth notwithstanding the larger litter sizes in this group. The replacement of soybean oil meal by full-fat soy flour causes the differences in litter size between the experimental and control groups to disappear (Jaffé, '55). They are therefore probably not related to vitamin B<sub>12</sub>.

The females on the deficient diet were significantly older when their first litters were born than those on the diet supplemented with vitamin B<sub>12</sub>. This is in accordance with observations of Dryden et al. ('54) on sexual maturation, which was found to be delayed in vitamin B<sub>12</sub>-deficient animals.

It can be concluded from the data of table 3, that the difference in growth, for the 4 weeks after weaning, between rats receiving vitamin B<sub>12</sub> supplements of 3 or 5 µg/kg of diet respectively, was small in both males and females (8.4 gm for males and 6.4 gm for females) as compared with the differ-



giving results which would indicate a somewhat higher requirement for vitamin B<sub>12</sub>.

The observations on rats presented in this paper are very similar to those made earlier on mice (Jaffé, '54), with the difference that in the latter species a supplement of 3 µg/kg of vitamin B<sub>12</sub> to the corn-soybean oil ration was as effective as the higher doses.

#### SUMMARY

A rat colony was kept on a fortified soybean oil meal-corn ration, low in vitamin B<sub>12</sub>, for 18 generations, using mostly brother and sister matings.

Litters starting with the second generation showed high mortality, low birth weights, low weaning weights, slow post weaning growth, and low liver and kidney vitamin B<sub>12</sub> levels. Females of this group were older, when giving birth to their first litters, than the controls. Blood characteristics and liver glutathione levels were normal.

No significant difference between succeeding generations could be detected and therefore no indication for a genetic selection toward resistance to vitamin B<sub>12</sub> deficiency could be found.

The addition of 3 µg of vitamin B<sub>12</sub> per kilogram of diet eliminated most of the deficiency symptoms, but did not result in optimal weaning weights and post-weaning growth, while supplements of 5 µg of vitamin B<sub>12</sub> per kilogram of diet, or 30 µg of this vitamin together with 0.2% of methionine, gave identical results in overcoming these deficiency signs. All of the animals used had been kept for at least one generation on the respective experimental diets previous to the experiments presented.

#### ACKNOWLEDGEMENT

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minous testimony placed in the record of the "bread hearings" (Federal Security Agency, '48-'49) was judged by the Administrator to be inadequate for the purpose of establishing the safety of Myrj 45. A substantial part of this toxicological evidence had been obtained in support of the use of various polyol esters of fatty acids in products other than bread, principally of a pharmaceutical nature. Much of the data had been accumulated prior to the publication by the Food and Drug Administration of its recommendations concerning the design of toxicological experiments to support the use of chemical additives in foods (Lehman et al., '49). However, the opinion of experts as expressed at these hearings was far from unanimous on the question of the inadequacy of the evidence particularly with respect to the chronic feeding studies. This fact, together with the cumulative experience of apparently safe pharmaceutical and food use of the polyol emulsifiers, prompted renewed investigation on the effect of chronic ingestion of the fatty acid partial esters.

Experiments were undertaken to determine the effects of these esters on growth, food utilization and metabolism, reproduction and lactation, physiological behavior, mortality, and post-mortem pathology, when included in the diet of rats throughout their lifetime, at levels far exceeding any conceivable use-concentration in the human diet. In the evolution of the plan for these experiments, careful consideration was given by all parties concerned to the magnitude and scope of the studies. In general the plan followed the pattern suggested for chronic feeding studies by the Division of Pharmacology of the Food and Drug Administration. The experiments were designed to cover successive generations of rats as well as the life cycle of the parent generation. The diets consisted exclusively of a nutritionally adequate basal ration modified only to the extent necessary by graded additions of the emulsifiers or of fat.

The studies are to be reported in a series of papers, this introductory report covering the general plan of the investigation and procedures which apply throughout, as well as

# BENEFICIAL EFFECTS OF ALFALFA ON THE OVARIAN DEVELOPMENT OF IMMATURE RATS FED MASSIVE DOSES OF ALPHA-ESTRADIOL<sup>1</sup>

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It is well established that the prolonged administration of massive doses of estrogens inhibits ovarian development in the immature rat (Zondek, '41). In the present communication data are presented indicating that the deleterious effects of massive doses of alpha-estradiol on ovarian development in the immature rat can be largely counteracted by the concurrent feeding of dried alfalfa. The protective factor (or factors) in alfalfa is apparently distinct from any of the known nutrients.

## EXPERIMENTAL

*Experiment 1. Comparative effects of dried alfalfa and supplements of the known nutrients on ovarian weight and morphology in immature rats fed massive doses of alpha-estradiol*

The basal ration employed in the present experiment consisted of sucrose, 66%; casein,<sup>2</sup> 24%; salt mixture,<sup>3</sup> 5% and

<sup>1</sup> Communication no. 401 from the Department of Biochemistry and Nutrition, University of Southern California.

<sup>2</sup> Vitamin-free test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>3</sup> Hubbell, Mendel and Wakeman salt mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

sumption and growth. However, it is not to be inferred that any of the emulsifiers, though they might have nutritional value, were regarded as equivalent to or substitutes for triglycerides. The neutral fat employed was a "pure vegetable shortening made from hydrogenated vegetable oil."<sup>3</sup>

*Experimental diets.* Since the ultimate objective of these experiments was to provide a basis for determining the safety of the use of the fatty acid partial ester emulsifiers in the human diet, special consideration was given to the choice of a basal diet for the chronic feeding studies.

Three types of basal ration are employed in chronic toxicological studies, the so-called synthetic or purified diets, commercially prepared feeding mixtures (mashes or chows), and laboratory-prepared diets composed principally of natural foodstuffs. The synthetic or purified type of diet was considered unsuitable for these experiments because of the more or less arbitrary selection of protein, carbohydrate, and fat components and the lack of variety as compared with the mixture of foods comprising the human diet. Furthermore the possibility of inducing effects due to the deficiency of unrecognized essential nutrients could lead to adventitious results in these chronic studies. Commercial mixes or chows were rejected on the ground that no direct or immediate control could be maintained over the composition or uniformity of such mixtures during the course of a prolonged experiment. Moreover the necessity for replacing high proportions of a whole prepared ration with test materials could lead to wide variations in the proportions of essential nutrients in the test and control diets.

For the foregoing reasons it was decided to employ a basal diet prepared in the laboratory primarily from natural foods, such as wheat, corn, milk, meat, etc., supplemented with various micronutrients. It was possible not only to insure the uniformity of composition but, even more important, to incorporate replacements of as much as 20% of esters or fat

<sup>3</sup> Primex, purchased in 50 lb. cartons at intervals, as required, from the Proctor and Gamble Distributing Company, Kew Gardens, New York.

discarded. These measures were employed to minimize the oxidative changes in the diet. Feeding was continued for 8 weeks. Animals were autopsied on the 56th day of feeding; ovaries were weighed and fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

TABLE 1

*Comparative effects of dried alfalfa, alfalfa fractions and supplements of the known nutrients on the ovarian weight of rats fed massive doses of alpha-estradiol*

SUPPLEMENTS FED WITH BASAL RATION	ALPHA- ESTRADIOL IN RATION	NUMBER OF ANIMALS	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	AVERAGE OVARIAN WEIGHT <sup>1</sup>
	mg/kg of diet		gm	gm	mg
<i>Experiment 1</i>					
None	10	20	42.8	177	18.6 ± 1.0
B vitamins, C and K	10	10	42.7	175	18.8 ± 0.9
Vitamins A, D and E	10	10	42.5	182	18.2 ± 1.6
5% Casein	10	10	42.4	199	19.1 ± 1.8
5% Corn oil	10	10	42.6	172	17.4 ± 1.2
2.5% salt mixture	10	10	42.3	176	19.3 ± 1.3
20% oven-dried alfalfa	10	10	42.2	184	34.2 ± 4.2
20% sun-dried alfalfa	10	10	42.2	175	41.6 ± 2.5
20% vacuum-dried alfalfa	10	10	42.1	175	40.8 ± 4.2
Basal ration without alpha-estradiol	0	10	41.9	209	53.4 ± 3.0
<i>Experiment 2</i>					
None	10	10	41.4	170	17.3 ± 1.1
20% alfalfa meal 1	10	10	41.2	185	19.4 ± 1.8
20% alfalfa meal 2	10	10	41.0	179	28.1 ± 1.7
20% alfalfa meal 3	10	10	41.2	184	34.2 ± 3.3
20% alfalfa meal 4	10	10	41.4	184	38.3 ± 3.9
20% alfalfa meal 5	10	10	41.1	185	38.1 ± 4.0
20% alfalfa meal 6	10	10	41.1	189	43.2 ± 2.1
Basal ration without alpha-estradiol	0	10	41.0	202	50.6 ± 1.8
<i>Experiment 3</i>					
None	10	10	41.0	155	14.5 ± 0.9
13% alfalfa residue	10	10	40.8	173	36.0 ± 4.0
7% dried alfalfa juice	10	10	40.9	175	33.7 ± 3.8
Basal ration without alpha-estradiol	0	10	41.0	185	46.5 ± 2.8

<sup>1</sup> Including standard error of the mean calculated as follows  $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.



The composition of the basal and supplemented diets is shown in table 1. Supplementation of the diet with esters or fat was accomplished by replacing equivalent amounts of the wheat-corn fraction which is present in the proportion of 2:1 in the basal diet, the other constituents of the diet remaining unchanged. Thus when an emulsifier was included to the extent of 20%, the protein content of the ration was only slightly affected, being reduced from 22 to approximately 20%, while the energy value varied within the narrow limits of 2.99 to 3.42 Cal. per gram (table 2).

TABLE 2  
*Caloric density of basal and test diets*<sup>1</sup>  
(All values in calories per 100 gm of diet)

SUPPLEMENT TO BASAL DIET	LEVEL, PER CENT			
	0	5	10	20
None	341.8			
Myrj 45		341.4	341.0	340.2
Myrj 52		331.1	320.4	299.0
Span 60		342.3	342.8	343.8
Tween 60		335.3	328.8	315.8
Tween 65		342.0	342.1	342.4
Tween 80		335.9	329.9	318.0
Mixture		339.0	336.1	330.4
Primex		369.0	396.2	450.6

<sup>1</sup> The caloric values of the emulsifiers are based on the assumption of 9.4 Cal. per gram for their fatty acid moieties, corrected for the observed coefficients of digestibility, viz. Myrj 45-3.32, Myrj 52-1.26, Span 60-3.50, Tween 60-2.09, Tween 65-3.42, Tween 80-2.21, Mixture—2.83 calories per gram, the last mentioned being the weighted average for the component emulsifiers.

Thiamine assays conducted on batches of the diet containing 0, 5, 10, and 20% of Myrj 45 showed no appreciable destruction of this vitamin after 6 weeks' storage at room temperature, in contrast with the finding of Nelson ('53). In any case completion of the diet batches by the addition of the emulsifiers, fat and vitamin mixtures was accomplished about once a week so that no diets were more than 10 days old when fed.

incorporated in the basal ration in place of an equal amount of sucrose. Animals were autopsied after 8 weeks of ad libitum feeding and ovarian weights determined. Results are summarized in table 1. In agreement with earlier findings the ovaries of rats fed the basal ration appeared immature both in weight and appearance. Considerable differences were observed, however, in the ovarian weight and appearance of rats fed the various alfalfa samples. One of the lots was virtually devoid of activity with corpora lutea present in only two of the 10 rats in the group. Three of the lots had moderate activity with corpora lutea present in 4 to 6 of the 10 rats in each group. The remaining two lots had high activity with all rats in each group exhibiting corpora lutea formation. In general the average ovarian weight of rats fed the various alfalfa fractions was directly correlated with the number of animals in each group with corpora lutea present.

*Experiment 3. Effects of dried alfalfa juice and alfalfa residue on ovarian weight and morphology of immature rats fed massive doses of alpha-estradiol*

Tests were conducted to determine the comparative effects of dried alfalfa juice and desiccated alfalfa residue<sup>7</sup> on the ovaries of immature rats fed alpha-estradiol. The experimental procedure was similar to that employed previously. Forty female Wistar rats comparable in age and weight to those employed above were divided into 4 groups of 10 rats each and were fed the following rations: (a) basal ration with alpha-estradiol omitted (b) basal ration containing alpha-estradiol (c) basal ration plus 13% of desiccated alfalfa residue and (d) basal ration plus 7% of dried alfalfa juice. Supplements were added in place of equal amounts of sucrose. Animals were autopsied after 8 weeks of ad libitum feeding and ovarian weights determined. Results are summarized in table 1. As in the previous tests the ovaries of rats fed the basal ration were immature both in weight and appearance.

<sup>7</sup> The water-washed alfalfa pulp remaining after the extraction of the juice.

random numbers (Cochrane and Cox, '50) litter-mates were assigned randomly to diets having the same level of supplementation. The groups consisted of more females than males because it was anticipated that the stresses of pregnancy and lactation might result in higher mortality, thereby leaving an insufficient number of females to establish statistically sound longevity data.

*Housing and maintenance.* For the entire duration of the investigation the rats were housed in suspended wire-mesh cages in an air-conditioned room maintained at  $76 \pm 2^{\circ}\text{F}$ . and  $50 \pm 10\%$  relative humidity. For the first 12 weeks on the test diets they were housed individually but when matings were started they were transferred to larger cages, one male being housed with one or two females. When litters were about to be cast the does were transferred to separate cages containing shredded paper wads for nesting where they remained until their litters were weaned. During rest periods between matings small groups of rats of the same sex were housed together.

Diets were furnished ad libitum in non-scatter food cups. Water was supplied through glass delivery tubes from bottles suspended on the outside of the cages. Cages were washed once a week or more frequently if they became soiled by soft stools.

*Observations.* During the first 12 weeks, the body weight of each rat was recorded at weekly intervals. This practice was followed not only in the initial generation but in each succeeding generation throughout the study. For the remainder of the experiment the animals were weighed biweekly. Records were made of food consumption of 5 rats of each sex, randomly chosen from each group, for the first 12 weeks. Additional checks on food consumption were made on these rats for a two-week period at the 0.5, 1, 1.5, and 2-year stages in the initial generation and for 12 weeks after weaning in succeeding generations.

At the termination of the 12-week period, 6 rats from each group (three males and three females) were randomly selected

Friedman reported the presence of a gonad-stimulating substance in extracts from alfalfa meal. This work was confirmed and extended in subsequent investigations by the same authors (Friedman and Friedman, '39). As was true in the present investigation, a great deal of variability was observed in the activity of different lots of test material (Friedman and Mitchell, '41). An unidentified water-soluble factor in cereal grass has been reported which is effective in promoting ovulation of rabbits sensitized with estrogen. This factor which differs in stability and in other properties from gonadotropins obtained from animal sources apparently exerted its effect by causing the release of pituitary gonadotropin (Borasky and Bradbury, '42; Bradbury, '44; Bradbury and Hodgson, '42). An unidentified factor in grass has been reported which produces early vaginal opening and stimulates early ovarian activity in immature rats. This material which has been referred to as the "sex maturity factor" was orally active, water soluble and was concentrated by alcohol precipitation of grass juice (Gomez et al., '41).

It has been demonstrated that the ovarian inhibition caused by the prolonged administration of estrogens in the immature rat is due to an impaired secretion of pituitary gonadotropins (Zondek, '41). The inhibition of ovarian development that occurs under these conditions can be counteracted by the concurrent administration of pituitary or chorionic gonadotropins. Inasmuch as desiccated alfalfa under conditions of the present experiment was also effective in counteracting the ovarian inhibition caused by the prolonged administration of an estrogen, the possibility exists that the protective effect of alfalfa was due to the presence of an orally active gonadotropin in this material, possibly comparable to the factor or factors indicated above. Another possibility is that alfalfa contains a factor (or factors) which was effective in promoting the synthesis and secretion of pituitary gonadotropin under conditions of the present experiment. An alternate interpretation is that alfalfa contains an unknown nutrient (or nutrients) which may be part of an enzyme system concerned

TABLE 3

Summary of growth responses of  $F_0$  generation of rats during two-year feeding experiment

FAT OR EMULSIFIER	NUMBER OF RATS AND SEX	AVERAGE BODY WEIGHTS, 0 TO 104 WEEKS								
		0	3	6	12	24	48	52	96	104
None	12M	gm	gm	gm	gm	gm	gm	gm	gm	gm
	20F	57	175	260	326	371	420	417	446 <sup>7*</sup>	431 <sup>7</sup>
		56	139	176	209	262	307	301	329 <sup>13</sup>	315 <sup>12</sup>
<i>5% Level</i>										
Myrj 45	12M	57	175	264	334	385 <sup>11</sup>	415 <sup>10</sup>	420 <sup>10</sup>	441 <sup>4</sup>	435 <sup>3</sup>
	20F	53	133	171	205	268 <sup>11</sup>	304 <sup>13</sup>	303 <sup>13</sup>	327 <sup>5</sup>	319 <sup>5</sup>
Myrj 52	12M	60	180	257	341	389	430	434	426 <sup>7</sup>	421 <sup>7</sup>
	20F	54	134	171	202	281	312 <sup>10</sup>	302 <sup>11</sup>	313 <sup>10</sup>	316 <sup>7</sup>
Span 60	11M	52	167	249	322	341	404	412	431 <sup>4</sup>	434 <sup>3</sup>
	20F	54	130	165	195	267 <sup>10</sup>	287 <sup>14</sup>	288 <sup>14</sup>	292 <sup>13</sup>	282 <sup>10</sup>
Tween 60	12M	57	162	243	320	373 <sup>11</sup>	424 <sup>11</sup>	424 <sup>10</sup>	450 <sup>5</sup>	455 <sup>5</sup>
	21F	55	133	169	200	256 <sup>10</sup>	290 <sup>13</sup>	288 <sup>13</sup>	317 <sup>10</sup>	292 <sup>9</sup>
Tween 65	12M	58	165	252	320	368	393	397	448 <sup>5</sup>	446 <sup>5</sup>
	20F	53	132	170	206	266 <sup>11</sup>	297 <sup>14</sup>	302 <sup>14</sup>	313 <sup>10</sup>	296 <sup>8</sup>
Tween 80	12M	56	170	246	310	354 <sup>11</sup>	393 <sup>11</sup>	383 <sup>11</sup>	412 <sup>4</sup>	423 <sup>5</sup>
	20F	54	140	179	210	260 <sup>11</sup>	303 <sup>17</sup>	311 <sup>17</sup>	317 <sup>11</sup>	311 <sup>7</sup>
Mixture	12M	55	171	257	327	378	421	416	428	416 <sup>12</sup>
	20F	57	137	177	209	264 <sup>13</sup>	303 <sup>15</sup>	307 <sup>15</sup>	315 <sup>9</sup>	326 <sup>8</sup>
Primex	12M	56	175	264	350	402	451 <sup>11</sup>	472 <sup>11</sup>	465 <sup>4</sup>	454 <sup>4</sup>
	20F	57	136	176	212	290	319 <sup>10</sup>	320 <sup>10</sup>	351 <sup>12</sup>	371 <sup>10</sup>
<i>10% Level</i>										
Myrj 45	12M	56	166	238	299	355	392 <sup>11</sup>	398 <sup>11</sup>	419 <sup>3</sup>	414 <sup>4</sup>
	20F	60	142	178	208	274 <sup>10</sup>	313 <sup>13</sup>	312 <sup>13</sup>	343 <sup>13</sup>	330 <sup>7</sup>
Myrj 52	12M	57	172	234	302	321 <sup>11</sup>	408 <sup>11</sup>	406 <sup>12</sup>	478 <sup>3</sup>	480 <sup>4</sup>
	20F	57	136	173	205 <sup>10</sup>	270 <sup>11</sup>	307 <sup>13</sup>	296 <sup>14</sup>	302 <sup>12</sup>	310 <sup>10</sup>
Span 60	10M	57	157	231	296	364	415	412	400 <sup>7</sup>	401 <sup>5</sup>
	20F	58	128	164	198	270	297 <sup>11</sup>	297 <sup>11</sup>	329 <sup>11</sup>	326 <sup>9</sup>
Tween 60	12M	58	170	238	308	373	423	419 <sup>11</sup>	444 <sup>9</sup>	438 <sup>8</sup>
	20F	57	135	174	206	272	302 <sup>11</sup>	300 <sup>11</sup>	337 <sup>9</sup>	323 <sup>5</sup>
Tween 65	12M	57	170	239	300	356	406	407	412 <sup>5</sup>	387 <sup>4</sup>
	20F	57	135	174	206	283 <sup>10</sup>	303 <sup>17</sup>	305 <sup>17</sup>	323 <sup>13</sup>	319 <sup>10</sup>
Tween 80	12M	58	159	242	305 <sup>10</sup>	375 <sup>10</sup>	409 <sup>10</sup>	414 <sup>9</sup>	438 <sup>5</sup>	387 <sup>5</sup>
	20F	57	137	177	209	270 <sup>10</sup>	325 <sup>11</sup>	306 <sup>11</sup>	332 <sup>12</sup>	315 <sup>10</sup>
Mixture	12M	56	160	235	301	360	413	410	445 <sup>8</sup>	450 <sup>3</sup>
	20F	55	132	172	203	272 <sup>11</sup>	314 <sup>16</sup>	310 <sup>15</sup>	338 <sup>13</sup>	334 <sup>10</sup>
Primex	12M	58	177	252	331	395	437	447	485 <sup>4</sup>	493 <sup>4</sup>
	20F	55	131	174	207	269 <sup>10</sup>	305 <sup>17</sup>	323 <sup>17</sup>	343 <sup>14</sup>	322 <sup>11</sup>
<i>20% Level</i>										
Myrj 45	12M	57	155	239	310	353 <sup>11</sup>	409 <sup>10</sup>	410 <sup>10</sup>	450 <sup>4</sup>	449 <sup>4</sup>
	20F	56	129	173	203	262 <sup>10</sup>	306 <sup>13</sup>	312 <sup>13</sup>	353 <sup>13</sup>	341 <sup>11</sup>
Myrj 52	12M	56	145	203	277	318 <sup>10</sup>	377 <sup>10</sup>	374 <sup>10</sup>	396 <sup>4</sup>	380 <sup>4</sup>
	21F	58	118	166	206	267 <sup>10</sup>	305 <sup>17</sup>	309 <sup>17</sup>	321 <sup>10</sup>	327 <sup>6</sup>
Span 60	12M	56	137	215	275	305 <sup>10</sup>	373 <sup>8</sup>	375 <sup>8</sup>	383 <sup>3</sup>	385 <sup>4</sup>
	20F	58	120	161	194	253 <sup>10</sup>	288 <sup>17</sup>	295 <sup>17</sup>	293 <sup>13</sup>	289 <sup>11</sup>
Tween 60	12M	58	133	212	280 <sup>11</sup>	334 <sup>11</sup>	388 <sup>10</sup>	397 <sup>10</sup>	407 <sup>6</sup>	404 <sup>4</sup>
	20F	58	118	172	202	274 <sup>10</sup>	300 <sup>17</sup>	310 <sup>17</sup>	316 <sup>5</sup>	333 <sup>5</sup>
Tween 65	12M	57	129	206	269	338 <sup>11</sup>	385 <sup>10</sup>	398 <sup>9</sup>	441 <sup>5</sup>	414 <sup>4</sup>
	21F	57	124	167	204	255	310 <sup>10</sup>	302 <sup>10</sup>	329 <sup>11</sup>	357 <sup>7</sup>
Tween 80	12M	58	141	205	277	351 <sup>10</sup>	388 <sup>8</sup>	388 <sup>8</sup>	426 <sup>4</sup>	422 <sup>6</sup>
	20F	57	117	166	200	270 <sup>10</sup>	300 <sup>14</sup>	307 <sup>14</sup>	332 <sup>5</sup>	340 <sup>6</sup>
Mixture	12M	58	150	218	281	339 <sup>10</sup>	395 <sup>10</sup>	387 <sup>10</sup>	440 <sup>5</sup>	409 <sup>6</sup>
	20F	59	129	172	202	256 <sup>10</sup>	305 <sup>14</sup>	311 <sup>14</sup>	332 <sup>12</sup>	320 <sup>10</sup>
Primex	12M	62	181	267	349	412			discontinued	
	20F	56	135	181	218	295				

\* Superscripts indicate number of survivors.

# THE INFLUENCE OF VITAMIN B<sub>12</sub> AND AUREOMYCIN UPON THE GROWTH OF PROTEIN- DEFICIENT CHILDREN

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The purpose of this study was to examine the influence of vitamin B<sub>12</sub> and aureomycin upon the growth of protein-deficient children and at the same time to make an assessment of their nutritional status. A brief preliminary report has been made (Mackay et al., '54).

There is disagreement as to the effect of dietary supplementation with vitamin B<sub>12</sub> on human growth and conflicting results have been reported (see for example Wetzel et al., '49, '52; Benjamin and Pirrie, '52; Scrimshaw and Guzman, '54). Studies with aureomycin have shown positive results (Snelling and Johnson, '52; Robinson, '52; Carter, '53). If such supplements should have a positive influence upon growth, their administration might make an important contribution towards alleviating the widespread malnutrition that exists in the tropics and which is present in the Caribbean area. It was for these reasons that the present study was undertaken.

The children upon whom the observations were made in the present study lived in small rural communities in the southern part of the parishes of St. Thomas and St. Andrews in Jamaica, B.W.I. The people of these districts earn a rather poor subsistence by agriculture. Some fishing is done but this

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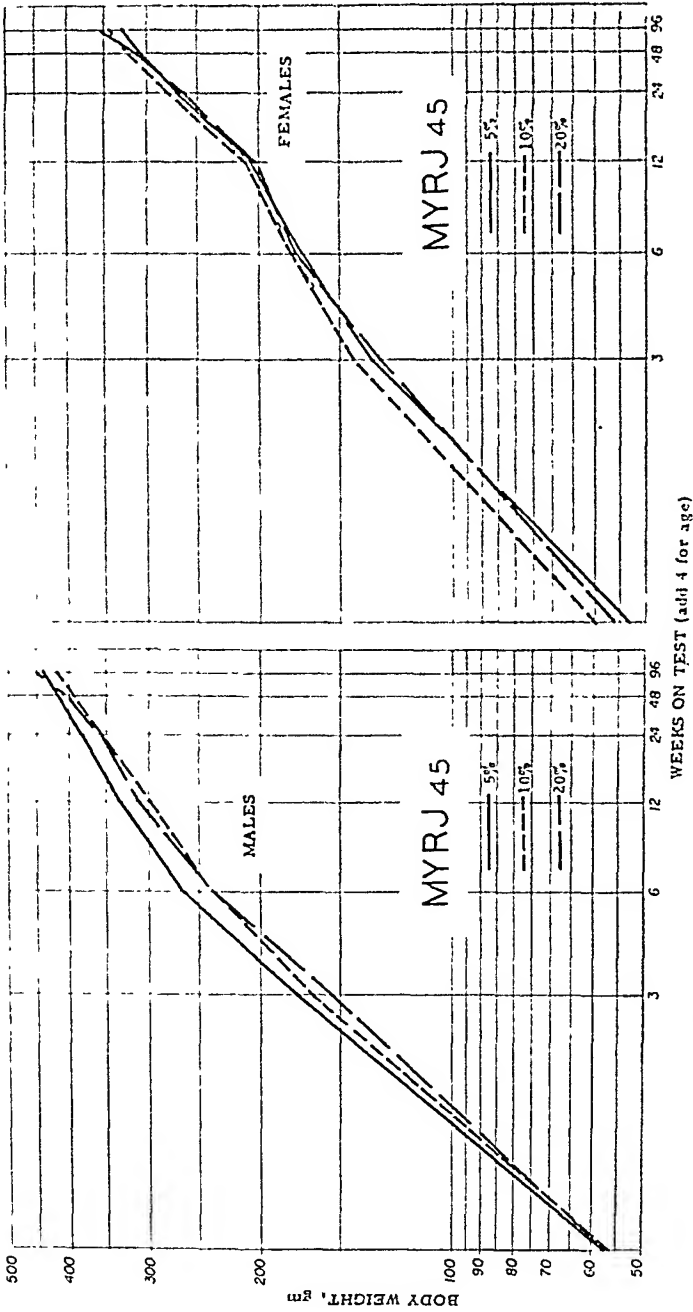


Figure 2

Figure 3

Fig. 2 and Fig. 3 Average growth curves of male and female rats, respectively, fed graded levels of Myrj 45.

The initial selection of the children to be used in the work on the evaluation of aureomycin and vitamin B<sub>12</sub> as growth promoters was based upon the use of the Wetzel grid. However, for the calculation of the final results, the assessment of growth was based upon increments of height and weight separately.

TABLE 1

*The average daily consumption per capita of the dietary supplements by Jamaican children*

GROUP	TROCHE	AV. DAILY CONSUMPTION	
		Vitamin B <sub>12</sub>	Aureomycin
		$\mu g$	$mg$
I	Placebo	0	0
II	Vitamin B <sub>12</sub>	65.3	0
III	Aureomycin	0	31.7
IV	Vitamin B <sub>12</sub> + aureomycin	64.0	32.0

TABLE 2

*The heights and weights of Jamaican children*

AGE	BOYS			GIRLS		
	Height		Weight	Height		Weight
<i>years</i>	<i>no.</i>	<i>inches</i>	<i>pounds</i>	<i>no.</i>	<i>inches</i>	<i>pounds</i>
4-5	6	41.8 $\pm$ 1.7 <sup>1</sup>	36.3 $\pm$ 3.1 <sup>1</sup>	9	40.6 $\pm$ 4.8 <sup>1</sup>	36.6 $\pm$ 8.9 <sup>1</sup>
5-6	15	44.6 $\pm$ 2.2	41.8 $\pm$ 6.3	15	42.9 $\pm$ 2.8	37.6 $\pm$ 4.3
6-7	17	46.1 $\pm$ 2.0	43.6 $\pm$ 3.9	22	45.0 $\pm$ 2.4	41.8 $\pm$ 6.1
7-8	101	46.5 $\pm$ 2.5	45.1 $\pm$ 7.1	98	47.2 $\pm$ 2.2	46.2 $\pm$ 7.1
8-9	157	48.5 $\pm$ 2.6	49.4 $\pm$ 7.1	159	48.9 $\pm$ 2.4	50.9 $\pm$ 6.9
9-10	161	50.2 $\pm$ 2.2	54.6 $\pm$ 8.2	180	50.9 $\pm$ 3.2	55.5 $\pm$ 8.3
10-11	146	51.8 $\pm$ 2.6	59.5 $\pm$ 7.4	173	52.2 $\pm$ 2.8	59.3 $\pm$ 9.3
11-12	144	53.7 $\pm$ 2.6	64.2 $\pm$ 8.3	137	54.3 $\pm$ 3.2	65.7 $\pm$ 11.2
12-13	128	55.7 $\pm$ 2.5	71.3 $\pm$ 4.1	122	56.8 $\pm$ 3.2	75.5 $\pm$ 13.7
13-14	107	57.4 $\pm$ 3.0	78.8 $\pm$ 11.8	111	58.6 $\pm$ 2.8	83.7 $\pm$ 14.6
14-15	85	58.5 $\pm$ 3.2	84.7 $\pm$ 13.4	68	59.4 $\pm$ 10.1	92.3 $\pm$ 14.1
15-16	14	60.5 $\pm$ 2.0	94.4 $\pm$ 12.5	14	61.1 $\pm$ 2.8	100.3 $\pm$ 14.7

<sup>1</sup> Mean and standard deviation.

The weight of the children was measured on a beam balance. As there is little seasonal variation in the warm climate of Jamaica, the clothes of the children were light in weight throughout the year, so the children were measured clothed, but without their socks and shoes.



Reference to table 4 shows that the male and female rats on the basal diet (containing no added fat or emulsifier) gained 269 and 153 gm, respectively, during the initial 12 weeks.

The average weight gains of the 5% emulsifier groups in 12 weeks ranged from 254 to 281 gm for the males and from 141 to 156 gm for the females. Statistical analysis according to the multiple range test of Duncan ('53) shows that none of these gains was significantly different (at the  $p=0.05$  level) from those of the basal control group. The only animals in the 5% emulsifier groups whose 12-week gain was significantly lower than the corresponding Primex animals, were the male rats on Tween 80.

The 12-week gains for the males at the 10% level of emulsifiers ranged from 239 to 250 gm, whereas the 10% Primex males gained 273 gm; the average gains for the corresponding females were 140 to 152 gm in the emulsifier groups and 152 gm in the Primex group. The differences between these gains in the emulsifier groups and either the control or Primex groups were likewise found not to be statistically significant according to the same criterion.

Increasing the dietary level of supplementation to 20% resulted in some growth depression in the emulsifier groups. The gain of 253 gm for the males on the 20% Myrj 45 diet was not significantly less than that of the basal controls. However the 12-week gains of the males in the remaining emulsifier groups ranged between 212 and 223 gm and were significantly lower. Among the females in the 20% groups, the range of weight gains on the emulsifier diets was 136 to 148 gm, the differences from the controls being not significant. The weight gains of the 20% Primex animals, both male and female, were statistically higher than those of the comparable control rats.

To sum up, it may be stated that at the 5 and 10% dose levels the differences in 12-week average net gain between the emulsifier groups and the controls were not statistically significant; and at the 20% level the gains in the emulsifier

fore the gross weight and height gains would not be an adequate measure of the response to the treatment.

For each individual, the rate of weight gain in pounds per month and the rate of height in inches per month were calculated by the method of least squares. An analysis of variance (Snedecor, '46) of these data was made for each of the two variables.

The conclusions which were indicated by the variance analysis are: (1) the rate of weight gain differs among the treatments; (2) The rate of height gain is not significantly

TABLE 4  
*Growth measurements of Jamaican children*

	AV. RATE OF WEIGHT GAIN POUNDS PER MONTH	AV. RATE OF HEIGHT GAIN INCHES PER MONTH
Control	$0.60 \pm 0.031^1$	$0.184 \pm 0.0049^1$
Vitamin B <sub>12</sub>	$0.56 \pm 0.031$	$0.175 \pm 0.0049$
Aureomycin	$0.67 \pm 0.032$	$0.178 \pm 0.0050$
Vitamin B <sub>12</sub> + aureomycin	$0.66 \pm 0.032$	$0.176 \pm 0.0050$
<i>Town</i>		
Yallahs	0.66	0.178
Maxfield Park	0.58	0.201
Aeolus Valley	0.69	0.168
St. Benedict	0.56	0.165
Grove	0.46	0.151
Bull Bay	0.75	0.146

<sup>1</sup> Standard error.

different from treatment to treatment; (3) Both the rate of weight gain and the rate of height gain differ among the towns; (4) The differences (or non-difference in the case of height) among the treatments are not affected by the differences among the towns.

The averages for the treatments and towns are shown in table 4.

Since *both* groups of children who received aureomycin showed greater rates of weight gain than the other groups, it seems probable that aureomycin had a significant positive effect. It must be pointed out, however, that the effect was

TABLE 5

Comparison of responses of four generations of rats (five of each sex per group) during 12-week feeding periods on test diets

PAT OR EMULSIFIER	GENERATION	AVERAGE NET GAIN			EFU <sup>1</sup> gm/100 gm food	ECU <sup>1</sup> gm/100 Cal.	AVERAGE FOOD INTAKE			EFU <sup>1</sup> gm/100 gm food	ECU <sup>1</sup> gm/100 Cal.	AVERAGE NET GAIN			EFU <sup>1</sup> gm/100 gm food	ECU <sup>1</sup> gm/100 Cal.	AVERAGE FOOD INTAKE			EFU <sup>1</sup> gm/100 gm food	ECU <sup>1</sup> gm/100 Cal.
		gm	gm/100 gm food	gm/100 Cal.			gm	gm/100 gm food	gm/100 Cal.			gm	gm/100 gm food	gm/100 Cal.			gm	gm/100 gm food	gm/100 Cal.		
None	F <sub>0</sub>	201	1248	4.71			193	1095	5.16			205	1224	16.7			191	1224	16.7		4.91
	F <sub>1</sub>	210	1333	4.62			202	1328	15.2			211	1385	15.2			207	1385	15.2		4.47
	F <sub>2</sub>	202	1342	4.42			213	1334	16.0			195	1414	13.8			207	1414	13.8		4.05
	F <sub>3</sub>	226	1394	4.74			214	1380	15.5			215	1519	14.2			221	1519	14.2		4.18
Myrj 45	F <sub>0</sub>	187	1176	15.9	4.66		193	1095	17.6		5.16	205	1224	16.7		5.16	191	1224	16.7		4.91
	F <sub>1</sub>	196	1307	15.0	4.39		202	1328	15.2		4.46	211	1385	15.2		4.46	207	1385	15.2		4.47
	F <sub>2</sub>	190	1305	14.6	4.28		213	1334	16.0		4.69	195	1414	13.8		4.69	207	1414	13.8		4.05
	F <sub>3</sub>	220	1353	16.3	4.77		214	1380	15.5		4.55	215	1519	14.2		4.55	221	1519	14.2		4.18
Myrj 52	F <sub>0</sub>	217	1289	16.8	5.13		189	1185	15.9		4.96	186	1350	13.8		4.96	214	1350	13.8		4.61
	F <sub>1</sub>	194	1268	15.3	4.62		205	1332	15.4		4.81	214	1492	14.2		4.81	207	1492	14.2		4.75
	F <sub>2</sub>	204	1322	15.4	4.65		196	1419	13.8		4.31	207	1501	13.8		4.31	207	1501	13.8		4.61
	F <sub>3</sub>	227	1438	15.8	4.77		206	1469	11.0		4.37	221	1675	13.2		4.37	221	1675	13.2		4.41
Span 60	F <sub>0</sub>	210	1189	17.7	5.17		188	1133	16.6		4.84	178	1116	15.9		4.84	181	1116	15.9		4.63
	F <sub>1</sub>	212	1406	15.1	4.41		195	1293	15.1		4.40	184	1372	13.4		4.40	194	1372	13.4		3.90
	F <sub>2</sub>	189	1248	15.1	4.41		205	1165	17.6		5.13	194	1302	14.9		5.13	194	1302	14.9		4.33
	F <sub>3</sub>	201	1403	14.3	4.18		208	1361	15.3		4.46	203	1552	16.3		4.46	203	1552	16.3		4.74
Tween 60	F <sub>0</sub>	210	1183	17.8	5.34		193	1152	16.8		5.11	188	1255	15.0		5.11	188	1255	15.0		4.75
	F <sub>1</sub>	198	1346	14.7	4.38		208	1416	14.7		4.47	189	1423	13.3		4.47	189	1423	13.3		4.21
	F <sub>2</sub>	212	1323	16.0	4.77		199	1177	13.5		4.10	181	1486	12.2		4.10	181	1486	12.2		3.86
	F <sub>3</sub>	233	1401	16.6	4.95		208	1411	14.7		4.47	202	1714	11.8		4.47	202	1714	11.8		3.74
Tween 65	F <sub>0</sub>	212	1187	17.9	5.23		189	1077	16.7		4.88	180	1123	16.0		4.88	180	1123	16.0		4.59
	F <sub>1</sub>	196	1249	15.7	4.59		208	1236	16.8		4.91	189	1396	14.4		4.91	189	1396	14.4		4.21
	F <sub>2</sub>	199	1346	14.8	4.33		209	1349	15.5		4.44	201	1380	14.8		4.44	201	1380	14.8		4.33
	F <sub>3</sub>	230	1416	16.2	4.74		242	1505	16.1		4.70	213	1594	13.4		4.70	213	1594	13.4		3.92
Tween 80	F <sub>0</sub>	218	1271	17.2	5.12		196	1130	17.3		5.25	190	1243	15.3		5.25	190	1243	15.3		4.79
	F <sub>1</sub>	198	1256	15.8	4.70		214	1391	15.4		4.67	229	1535	14.9		4.67	229	1535	14.9		4.69
	F <sub>2</sub>	203	1398	14.5	4.32		202	1379	14.6		4.43	211	1429	14.8		4.43	211	1429	14.8		4.65
	F <sub>3</sub>	204	1462	14.0	4.17		224	1426	15.7		4.76	235	1681	14.0		4.76	235	1681	14.0		4.40
Mixture	F <sub>0</sub>	206	1190	17.3	5.10		196	1119	17.5		5.21	187	1200	15.6		5.21	187	1200	15.6		4.72
	F <sub>1</sub>	218	1389	15.7	4.63		201	1379	14.6		4.34	207	1422	14.6		4.34	207	1422	14.6		4.42
	F <sub>2</sub>	206	1355	15.2	4.48		224	1442	15.5		4.61	226	1420	15.9		4.61	226	1420	15.9		4.81
	F <sub>3</sub>	212	1268	16.7	4.93		211	1413	14.9		4.43	235	1512	13.0		4.43	235	1512	13.0		3.94
Primex	F <sub>0</sub>	226	1182	19.1	5.18		217	1044	20.8		5.25	234	1031	22.7		5.25	234	1031	22.7		5.04
	F <sub>1</sub>	219	1239	17.7	4.80		216	1284	16.8		4.42	231	1254	18.4		4.42	231	1254	18.4		4.08
	F <sub>2</sub>	185	1162	15.9	4.31		192	1098	17.5		4.42					4.42					
	F <sub>3</sub>	188	1217	15.4	4.17		203	1208	16.8		4.24					4.24					

<sup>1</sup> EFU = efficiency of food utilization; ECU = efficiency of substrate utilization.

discriminated

<sup>1</sup> EFU = efficiency of food utilization; ECU = efficiency of caloric utilization.

and palpable liver. The definition of these signs in general followed that of Abbott ('50), Burgess and Laidin ('50), and Jelliffe and Williams ('54). The picture which emerged from this work was that of marginal malnutrition; for example chelosis was found in 5% of the population, dry rough skin on the legs in 40%, tongue abnormalities in 20% and a palpable liver in 10%. There appeared to be no important differences in the clinical signs following the administration of the supplements. Particular attention was paid to evidence of toxicity resulting from the administration of the vitamin B<sub>12</sub> or aureomycin, but no such evidence was observed.

*Parasitic infestations.* Through the courtesy of Dr. Oscar Felsenfeld and his group from Chicago an assessment was obtained for the incidence of parasitological infestation of these children.

A low incidence of infestation with hookworm (1.5%) and ascaris (2.6%) was found. The incidence of *Trichuris trichura* (4%) and *Guardia lamblia* (14%) was greater, although, in comparison with African communities, it is very low indeed (McGregor and Smith, '52). This is probably due to the efficient and continuous regular treatment by the Public Health Authorities. The children in Jamaican school receive at regular intervals treatment for intestinal parasites. This treatment by the Public Health Authorities was a continuous and constant factor and was not altered during the period of this study. No antibiotics were given by the Public Health Authorities. There were no important differences in the infestation rates amongst the 4 groups of children. It is interesting to note that in 39% of the cases, the stools contained large amounts of undigested food.

Malaria officers of the Island Medical Service kindly examined the schools in our area during the time of our survey. The children were examined for enlargement of the spleen. The blood smears were taken from all of those with enlarged spleens and random samples of others. Of 698 children examined, 18.2% showed splenic enlargement and 27.0% of all blood smears taken were positive.

nificant when the differences in caloric density of the diets were taken into account. However even after such adjustment was made, the variances due both to levels of the emulsifiers and generations proved to be significant.

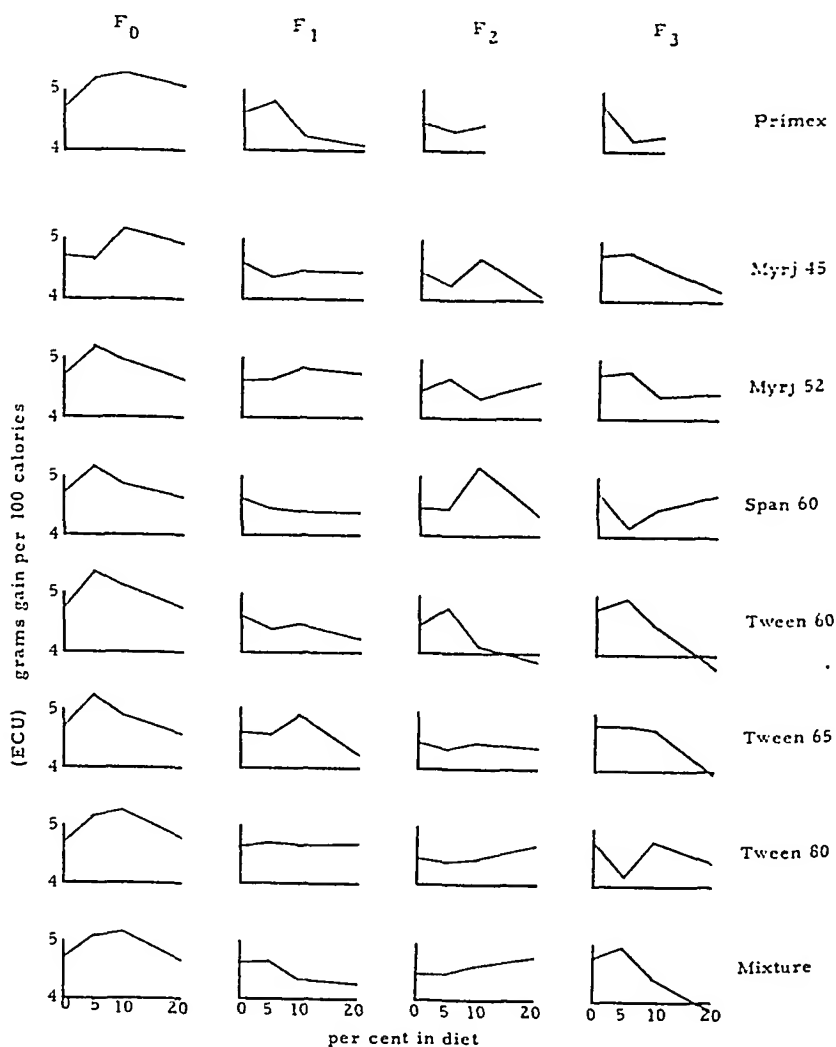


Fig. 4 Changes in efficiency of caloric utilization (ECU) with increasing dietary level of emulsifiers or Primex in 4 successive generations of rats.

from North American subjects but there appears to be comparatively less albumin and more globulin in the Jamaican children. A group of 31 normal adults, not West Indians, but living in Jamaica, was found to have an average concentration of serum protein of 7.12 gm % and an average concentration of serum albumin of 4.25 gm %. The hemoglobin concentration appears to be definitely below average values for children from other areas of the world (Hawkins and

TABLE 5

*Blood constituents in the groups of children after dietary supplementation*

CONSTITUENT		CONTROL (Group I)	VITAMIN B <sub>12</sub> (Group II)	AUREOMYCIN (Group III)	AUREOMYCIN + VITAMIN B <sub>12</sub> (Group IV)
Total serum	No.	52	47	50	49
protein, gm %	Mean	7.40	7.35	7.29	7.37
(Gradient tube)	S.D.	0.32	0.41	0.51	0.40
Total serum	No.	52	47	50	49
protein, gm %	Mean	7.39	7.33	7.24	7.33
(Colorimetric)	S.D.	0.32	0.39	0.43	0.41
Serum albumin,	No.	52	47	50	49
gm %	Mean	3.85	3.71	3.62	3.76
(Colorimetric)	S.D.	0.36	0.39	0.32	0.34
Haemoglobin,	No.	52	47	50	49
gm %	Mean	11.44	11.69	11.91	11.29
	S.D.	1.7	1.8	1.4	1.5
Serum cholin-	No.	16	20	16	15
esterase,	Mean	51	53	50	49
Q <sub>Co2</sub>	S.D.	8	9	11	9

Kline, '50). The non-West Indian group of adults (15 males and 16 females) had an average of 14.4 gm % hemoglobin.

Measurements of the concentration of vitamin B<sub>12</sub> after administration of a test dose of this substance have been reported elsewhere (Patrick, '55). It was found that 19 of the children from group I had an average serum concentration of vitamin B<sub>12</sub> of 0.21 µg/ml, in close agreement with the values quoted for healthy North American adults (Rosenthal and Sarett, '52). Nineteen of the children from group II had

indicated by the dual lines for this emulsifier in table 6 showing that the ECU values for the  $F_0$  generation, while not significantly higher than for  $F_1$ , were higher than for  $F_2$  and  $F_3$ .

In the case of the Span 60 series it is interesting to recall that the growth response in the  $F_0$  generation was somewhat

TABLE 7

*Grouping of ECU values according to statistical significance of differences among generations and dietary levels<sup>1</sup>*

	GENERATIONS				DIETARY LEVELS		
	$F_0$	$F_1$	$F_2$	$F_3$	5%	10%	20%
Myrj 45	—	—	—	—	—	—	—
Myrj 52	—	—	—	—	—	—	—
Span 60	—	—	—	—	—	—	—
Tween 60	—	—	—	—	—	—	—
Tween 65	—	—	—	—	—	—	—
Tween 80	—	—	—	—	—	—	—
Mixture	—	—	—	—	—	—	—
Primex <sup>2</sup>	—	—	—	—	—	—	—

<sup>1</sup> Generations or levels not included in the continuous lines are significantly different ( $p = 0.05$ ) from those so included, according to Duncan's multiple range and multiple F tests.

<sup>2</sup> 0, 5 and 10% Primex groups, the latter two being included to provide orthogonality.

lower than that in the other emulsifier groups; however this inferior performance was not seen in the subsequent generations nor was it evident in the data for ECU.

The most probable explanation for the generally higher ECU values in the first generation rats is that when placed on the test diets the  $F_0$  animals' prenatal and preweaning nutrition was qualitatively, if not quantitatively, different

TABLE 6

*Average daily per capita intake of various nutrients by Jamaican children*

SCHOOL	NUMBER STUDIED	AVERAGE AGE	CALORIES	PROTEIN	FAT	CARBOHYDRATE	CALCIUM	IRON	VITAMIN A	THIAMINE	RIBOFLAVIN	NIACIN	ASCORBIC ACID	PHOSPHORUS	RATIO OF ANIMAL TO VEGETABLE PROTEIN
		YRS. mos.		gms	gms	gms	mg	mg	I.U.	mg	mg	mg	mg	mg	
Aecobs Valley	43	10 11	1952	44.8	21.3	230	265	13.1	1848	0.730	0.528	6.09	142	606	1:2.5
August Town	7	10 8	1733	49.8	43.6	286	390	16.9	1063	0.953	0.829	7.77	73	637	1:1.7
165 Bull Bay	17	10 10	1621	45.8	29.9	289	242	12.5	1396	0.932	0.599	8.52	131	628	1:2.8
St. Benedict's	31	12 11	1591	46.5	32	279	215	9.9	1352	0.963	0.639	7.95	46	568	1:2.3
Grove	21	11 6	1671	49	37	289	286	14.1	2781	0.962	0.768	9.71	172	639	1:1.9
Yallahs	47	11 1	1546	46	32	276	308	14.7	1447	0.961	0.706	7.99	151	621	1:1.8
Average of above		11 4	1686	47	32	276	284	13.5	1648	0.917	0.678	8.01	119	617	1:2.16
NRC recommended allowance		10-12 yrs													
(Modified for tropical use)		78 lbs	2313	70			1100	12	4500	1.20	1.900	14.7	75		1:1.0
Maxfield Park 4-7 years			1561	47.5	46.5	240.5	747	10.5	2048	0.908	1.356	6.43	50	860	
7-11 years			2006	60.5	65	297.5	613.5	13.67	2218	1.179	1.312	8.57	68	889	



Myrj 45, Myrj 52, Span 60, Tween 60, Tween 65, Tween 80 and a mixture thereof. The parent generation consisted of 810 rats. Three successive generations comprising 1440 additional rats were likewise observed for growth, food efficiency and reproductive performance.

Observations were made of body weight and food consumption permitting estimates of the efficiency of food utilization; hemocytology and hemochemistry; physical appearance and behavior; water consumption; laxation; metabolic utilization of the partial esters; reproduction and lactation mortality rates; and gross and microscopic pathology. The findings and conclusions are to be reported in a series of papers of which this is the first.

The growth responses of the emulsifier groups at the 5 or 10% levels were not significantly different from the controls. The only emulsifier group at these levels showing a significantly lower gain than the corresponding Primex group were the males on 5% Tween 80. At the 20% level the males (but not the females) in all emulsifier groups except Myrj 45 showed a moderate but statistically significant reduction in weight gain as compared with the controls; the 20% Primex group gained even more than the controls.

Since food consumption records were maintained for the first 12 weeks of the test and the growth curves plotted on a log weight: reciprocal age basis were essentially linear, extensive statistical analyses were made for this initial period. The data reveal a trend in the direction of higher food intake with increasing emulsifier level whereas the opposite was noted in the Primex groups whose diets, in contrast with the emulsifier diets, increased substantially in caloric density as the fat level increased. Regardless of the test supplement fed, the efficiency of food utilization (EFU) as well as the efficiency of caloric utilization (ECU) were somewhat higher in the initial ( $F_0$ ) generation than in the succeeding generations. This may be explained by the difference in nutritional reserves at weaning between the first generation and all the descendent generations.

the influence of these factors in human subjects should be undertaken as, should a positive effect be observed upon growth it might provide us with a means of alleviating malnutrition. The obvious way to alleviate malnutrition is to remedy all of the dietary deficiencies but this is not always a practical possibility in tropical areas such as the Caribbean, and therefore the importance of examining any suggestions for a solution to nutritional problems cannot be over-emphasized. For this reason this study was undertaken.

The results obtained here suggest that aureomycin has a slight positive effect on weight gain, but no effect on height gain in these children. There was no positive effect observed from the administration of vitamin B<sub>12</sub>, but it should be emphasized that for the practical purpose of alleviating malnutrition these results are disappointing and it is unlikely that such factors would be of any practical value in dealing with the problem of growth failure where marginal malnutrition exists.

It might be that more obvious effects would be observed in a population which was more chronically and severely undernourished than that in Jamaica. The low intake of animal protein at first suggested that the children may have been deficient in vitamin B<sub>12</sub> but the estimations of serum B<sub>12</sub> concentration failed to confirm this.

The estimation of nutritional status where the malnutrition is only marginal is a difficult assessment and we have yet to find a satisfactory index for this measurement. We had thought that growth measurement might be most helpful, and this may be the case in large groups of children, but in the individual case it is probably of little value.

The study is self-consistent; that is, it was run in several towns and the results are essentially the same in all the towns. Since there is this high degree of internal consistency we believe that the explanation of the disagreement of the results here with other studies must be due to factors which distinguish the children in Jamaica from the children who were subjects of the other studies.

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In recent years advances in biochemical techniques have made it possible to increase the number of metabolic responses that can be observed simultaneously. This investigation was planned to explore possible biochemical differences in the metabolism of fat and carbohydrate among women of average, above and less than average body fat through simultaneous respiration studies and analyses of various blood and urinary constituents before and following test meals high in fat or carbohydrate.

TABLE 1  
*Physical description of subjects*

	SUBJECTS		
	Underweight	Average weight	Overweight
No.	7	7	7
Age, years			
Range	25 to 51	25 to 52	25 to 57
Mean	36	36	40
Height, cm			
Range	154.5 to 168.0	157.0 to 173.0	161.5 to 167.8
Mean	163.4	164.8	164.5
Weight, kg			
Range	46.0 to 52.5	53.5 to 69.9	76.2 to 112.0
Mean	49.8	60.9	87.5
Percentage deviation from desirable weight			
Range	- 12 to - 21	- 10 to + 13	+ 21 to + 66
Mean	- 15.5	- 0.5	+ 35.5

#### EXPERIMENTAL PLAN

Twenty-one women, from 25 to 57 years of age, were subjects (table 1). All were in good health and physically active; 7 were married and 6 had had children. The subjects were grouped into three classes: overweight, greater than 15% above; average weight, within + 15 to - 10% of; and underweight, more than 10% below desirable weight. The desirable weight for each subject was estimated from tables of the

# ABSORPTION OF TOPICALLY APPLIED VITAMINS

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## TWO FIGURES

It is well known that in ulcerative colitis, obstructive jaundice, enteritis and various non-specific diarrhoeas and dysenteries, absorption of essential nutrients from the intestinal tract may be greatly impaired. This condition may be associated with a weakening of the metabolic body processes due to the lack of absorption of the essential dietary nutrients.

Medical literature contains reports of the failure of patients to respond to either the oral or parenteral administration of nutrients and drugs. Vilter et al. ('53) demonstrated that pyridoxine when applied topically in an ointment was effective in the treatment of seborrheic dermatitis of the sicca type, whereas oral or parenteral treatment was ineffective. It was reported by Dainow ('52) that niacin topically applied to the skin lesions of pellagrins caused a dramatic response in healing of the lesions. This type of lesion requires a long period of oral therapy to produce complete remission. Sobel and Rosenberg ('53) stated that topical application of vitamin A was nearly as effective in overcoming vitamin A deficiency in rats as when the vitamin was given orally.

The present work is an extension of experiments which were recently the subject of a preliminary report (Greene et al., '54). Due to the comparative ease with which vitamin de-

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A fasting capillary blood sample was taken from the finger and sampled immediately for glucose and pyruvic acid analyses; fasting capillary serum was obtained for chylomicron counts and analysis of total lipids. A fasting urine collection was made preceding the administration of the meal.

Capillary blood samples were obtained one-half, one, two, three, 4, and 5 hours after the subject had finished the test meal. Blood glucose concentrations and serum chylomicron counts were determined for each blood sample; pyruvic acid and serum total lipid concentrations were determined for the one-, three- and 5-hour samples only. The volume of expired air was measured for single 8-minute periods at one, three and 5 hours after the test meals and sampled for gas analyses. The subject was at bed rest preceding each measurement of expired air. Urine samples were obtained at one, three and 5 hours after completion of the test meals. The subjects were in the laboratory during the entire test period. They were allowed to converse, read, write or do hand sewing except for the periods of bed rest. No food other than the test meal was consumed and water was permitted only in small amounts at the end of each expiratory air sampling period; there was no smoking.

*Analytical methods.* The metering device of the Kofranyi-Michaelis respirometer was calibrated and correction factors for absorption and diffusion of carbon dioxide and oxygen in the rubber collection bag were established. Samples of expired air were analyzed for carbon dioxide and oxygen by the conventional Haldane-Henderson-Bailey method (Peters and Van Slyke, '32). Overall respiratory quotients, total oxygen consumption, non-protein respiratory quotients and total heat production were calculated according to Brody ('45). The Zuntz non-protein respiratory quotient factors were used for converting oxygen consumption to calories per liter. When the determined non-protein respiratory quotients were greater than 1.00 or less than 0.70, the Zuntz factors for 1.00 and 0.70, respectively, were used. DuBois ('36) has shown that the error of such a procedure is negligible. Energy

the rats were confined in a tight-fitting wire enclosure to prevent any access to the ointment with their feet or tongues. During this period food and water were withdrawn. After the absorption interval, the area of ointment application was washed with soap and water and then with alcohol. At the times indicated in the tables the urinary excretion of the vitamins was measured. For this, the rats, after treatment as above, were placed in metabolism cages with food removed and a 16-hour urine sample collected for assay.

To demonstrate that the results obtained are a measure of skin absorption and not of rupture of the epidermal skin layer during denuding, 100 times the lethal dose of curare was administered in a manner similar to that used for the vitamins. None of the animals so treated exhibited any symptoms of curare poisoning.

#### RESULTS

##### *Transfer of vitamins through skin by topical application of thiamine*

To test the efficacy of different ointment bases or diluents as vehicles for topical application of individual vitamins, Plastibase Hydrophilic<sup>5</sup> and a Carbo-wax ointment base CMC-120,<sup>6</sup> were compared with a 3.75% solution of vanillin in 25% alcohol. Ten milligrams of ointment were applied to the denuded area, dosage of the vitamin being as indicated in table 1. The results of a typical experiment indicate that Plastibase Hydrophilic is an excellent medium for absorption of topically applied thiamine and is superior to either Carbo-wax CMC-120 or vanillin solution. In view of the small dosage of thiamine applied to the skin, and the fact that only two hours was allowed for absorption, the utilization of the topically applied thiamine as measured by body weight gain and urinary excretion is considered highly efficient. Rats hav-

<sup>5</sup> Plastibase Hydrophilic is a Squibb Trade name. The preparation used was an oleaginous type of base, consisting of 5% polyethylene and 95% mineral oil, to which 5% of glycerol monooleate was added to render it hydrophilic.

<sup>6</sup> This ointment is a water-soluble mixture of polyethylene glycol and carboxymethyl cellulose.



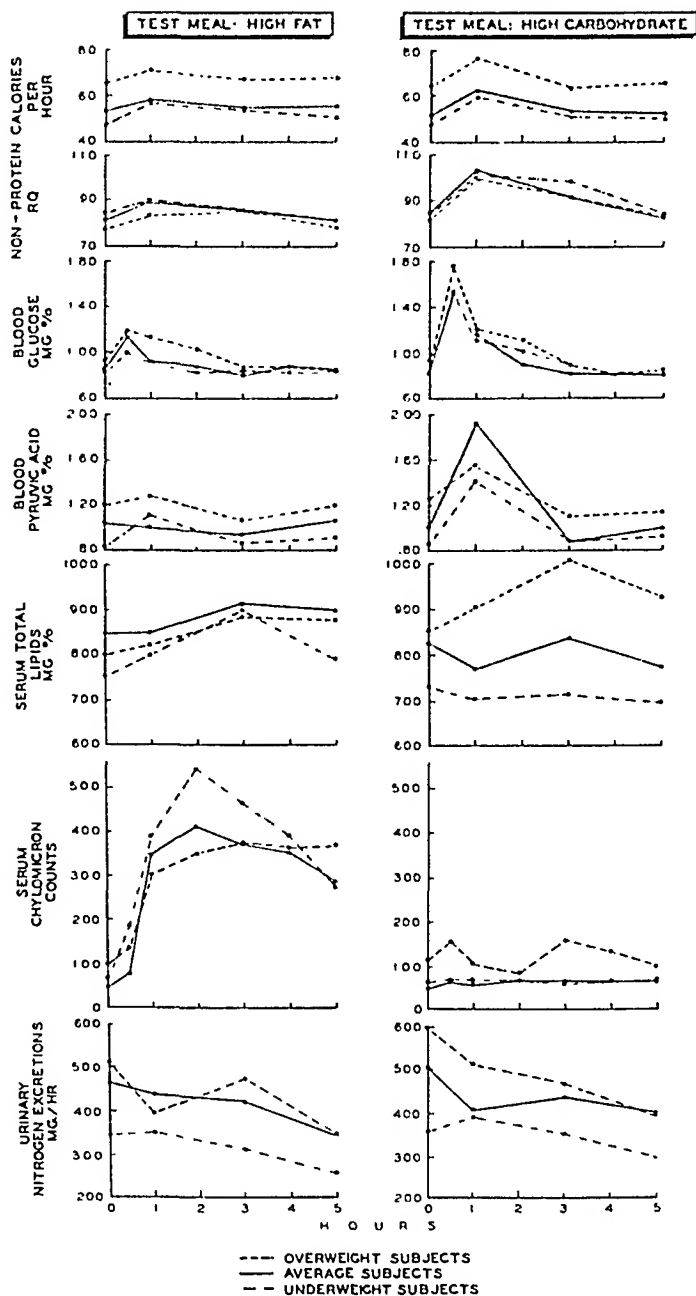


Fig. 1 Summary of mean metabolic patterns for underweight, average weight and overweight subjects.

ing symptoms of polyneuritis treated in this manner rapidly recovered and gained in body weight at a normal rate.

*Riboflavin.* The utilization of riboflavin after topical application is illustrated by the data in table 2. The results, as measured by body weight gain and urinary excretion, indicate that either Plastibase Hydrophilic or vanillin solution is a satisfactory vehicle for the adsorption of riboflavin. The dose of 500  $\mu$ g of riboflavin administered two times per week in the Plastibase Hydrophilic was not quite as efficient as

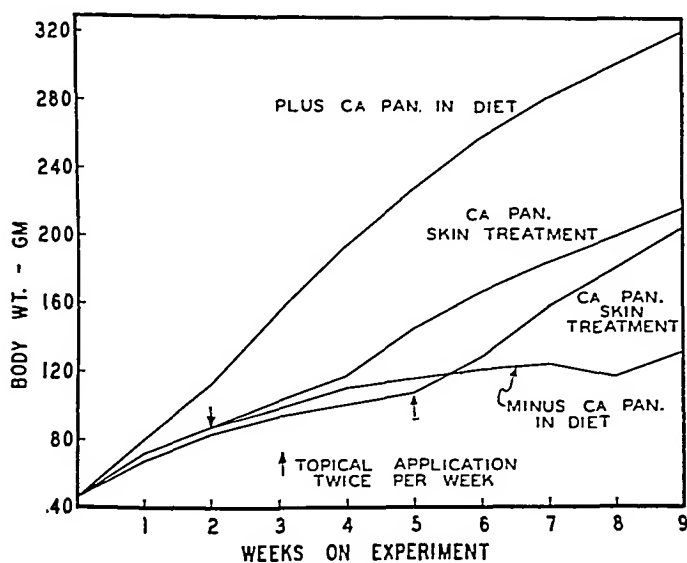


Fig. 1 Skin absorption of calcium pantothenate.

250  $\mu$ g fed orally. As was noted in the absorption of thiamine, Carbo-wax CMC-120 as a vehicle for riboflavin is inferior to either Plastibase Hydrophilic or vanillin solution.

*Pantothenic acid.* Unpublished experiments in this laboratory using hooded rats fed a semipurified complete diet, with only calcium pantothenate omitted, resulted in a gradual cessation of growth accompanied by a greying of the dark hair in two to 5 weeks. Addition of calcium pantothenate (20 mg per kilogram of diet) to the above deficient diet prevented this syndrome, or cured the syndrome after its manifestation.

ments in energy expenditures after the high-fat test meal were significantly higher for the underweight subjects than for the average and overweight subjects; differences among groups following the high-carbohydrate test meal were not significant. Further, the mean caloric expenditure for the underweights was less after the high-carbohydrate test meal than after the high-fat test meal, whereas the majority of the overweight and average weight subjects showed the greater caloric expenditure following the high-carbohydrate test meal.

The observed dynamic effect of food may be affected by the nutrient combination in which it is metabolized, the method of disposition of absorbed nutrients by the individual and by the technique of measurement. The differences in energy expense following the test meals in this study suggest that the underweight subjects may have had a metabolic pattern different from that of the average or overweight subjects, although the caloric differences were quantitatively small.

The mean fasting non-protein respiratory quotients for the underweight, average weight and overweight subjects were 0.83, 0.83 and 0.79, respectively; the differences were not statistically significant. Following the high-carbohydrate test meal, the changes in mean respiratory quotients of the overweight and average weight groups were similar; the underweights had a delayed decrease in respiratory quotient from peak values. In contrast, peak responses were reached less quickly for the overweight subjects than for the others after the high-fat test meal.

*Blood glucose and pyruvic acid.* There was a highly significant relationship between the fasting glucose concentrations in capillary blood and the percentage deviations from desirable weight (fig. 2). Eleven of the 14 fasting values for the 7 overweight subjects were between 90 and 101 mg/100 ml of blood, whereas only three fasting values for the average weight and one for the underweight subjects equalled or exceeded 90 mg/100 ml. Previous studies indicating that fasting blood glucose concentrations were higher among overweight than among average weight subjects have been clinical

In figure 2, the result of the topical application of 20 mg of pyridoxine, twice per week, in a 3.75% solution of vanillin, is shown. Topical application was made at the point where the weight of the rats plateaued for three days, and when scaly lesions on the front feet and about the mouth appeared. Increase in weight with a gradual disappearance of the scaly lesions in 14 days resulted. The resumption of growth and the cure of acrodynia symptoms show that the pyridoxine was effectively transferred through the skin.

In the pyridoxine and calcium pantothenate experiments, about 2 cm<sup>2</sup> of hair was removed from the scapular region,

TABLE 3

*Effect of topically administered vitamin D to vitamin D-deficient rats*

TEST DOSE U.S.P. UNITS	NO. OF RATS	METHOD OF DOSING	AVERAGE DEGREE OF HEALING
5	9	Alcoholic solution, topical	5.44 +
10	10	Alcoholic solution, topical	6.60 +
20	9	Alcoholic solution, topical	9.00 +
40	10	Alcoholic solution, topical	11.00 +
5	10	Plastibase hydrophilic, topical	4.20 +
10	9	Plastibase hydrophilic, topical	7.10 +
20	10	Plastibase hydrophilic, topical	8.20 +
40	10	Plastibase hydrophilic, topical	10.80 +
3.2	10	Standard D oil, oral	4.55 +
4.5	9	Standard D oil, oral	5.33 +

then the vitamin was applied by dropping the dose from a calibrated syringe. The rats were immediately immobilized in a wire enclosure for two hours. After the absorption period the rats were removed from the enclosure and the region of topical application was thoroughly washed.

*Vitamin D.* In the vitamin D experiments, doses of calciferol theoretically containing 40,000,000 U.S.P. units of vitamin D per gram were taken up in a 20% alcoholic solution or mixed in Plastibase Hydrophilic. The effect of administering vitamin D topically was compared to feeding vitamin D orally as prescribed by the U.S.P. XIV line test ('50), where the standard reference sample contained 400 U.S.P. units of vita-

to the higher mean fasting values in this group; the increments of elevation above fasting concentrations were higher, but not significantly higher, among the overweight subjects than among the average or underweight subjects. On the other hand, the mean blood glucose concentrations of the overweight subjects were significantly lower than fasting concentrations at the 4th and 5th hours; this response was not observed among the other two groups.

Following the high-carbohydrate test meal, there were no significant group differences in blood glucose concentration until the second hour, although the mean values for the overweight group were consistently higher than those of the other groups. At the second hour the mean blood glucose concentration of the overweight subjects was significantly higher than that of the average weight subjects but not of the underweight subjects. This higher concentration of the overweight group again was related in part to their higher fasting concentrations. And again at the 4th and 5th hours the blood glucose concentrations of the overweight subjects were below their fasting concentrations. The decrease in blood sugar was particularly rapid in view of the higher concentration for this group at the one-half hour period. Thus a distinct pattern of response was observed among the overweight subjects in the decrease of blood glucose concentrations below fasting values at the 4th and 5th hours following both test meals. The occurrence of greater post-absorptive hypoglycemia following carbohydrate feeding among obese than among "normal" subjects has been observed previously (Ogilvie, '35).

Mean fasting concentrations of blood pyruvic acid were 0.85, 1.02 and 1.22 mg/100 ml of blood for the underweight, average weight and overweight subjects, respectively. The mean fasting concentration reported by Meyer and Winkler ('52) for underweights was 0.89 and for overweights, 1.08 mg/100 ml.

Following the high-fat test meal, there was little change in blood pyruvic acid concentrations for any subject from

## SUMMARY

The absorption of topically applied vitamins by the rat has been determined. In view of the small doses of the vitamins (thiamine, riboflavin, pantothenic acid, pyridoxine and vitamin D) administered and the limited time allowed for absorption, the utilization of the vitamins studied is considered highly efficient. In addition to the body weight gain responses, and the prevention or cure of deficiency symptoms, the data on urinary excretion of thiamine and riboflavin substantiate the conclusion.

In general, a plasticized hydrocarbon water-absorbing gel ointment base was superior to a carbowax preparation as a vehicle for skin transfer of the vitamins studied. An alcoholic vanillin solution was also an effective vehicle for the skin absorption of riboflavin, pantothenic acid, pyridoxine and vitamin D.

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TABLE 3

*Mean fasting serum lipid constituents*

SERUM LIPID CONSTITUENT	SUBJECTS		F-VALUE <sup>1</sup> Analysis of variance Total	
	Underweight	Average weight		Overweight
Total lipids, venous, <sup>2</sup> mg/100 ml	795 ± 46.3 <sup>3</sup>	685 ± 62.6 <sup>3</sup>	796 ± 50.0 <sup>3</sup>	0.25
Total lipids, capillary, <sup>4</sup> mg/100 ml	750 ± 42.5	808 ± 76.5	826 ± 35.3	0.41
Total cholesterol, venous, mg/100 ml	214 ± 12.7	253 ± 19.7	228 ± 9.6	0.90
Lipoprotein fractions, venous: <sup>5</sup>				
S <sub>1</sub> 12-20, mg/100 ml	34 ± 6.8	18 ± 3.3	38 ± 13.1	1.37
S <sub>2</sub> 21-35, mg/100 ml	14 ± 3.8	10 ± 3.0	14 ± 6.8	0.21
S <sub>3</sub> 35-100, mg/100 ml	29 ± 9.1	22 ± 6.9	31 ± 14.0	0.22
S <sub>4</sub> 12-100, mg/100 ml	78 ± 17.5	51 ± 12.0	83 ± 33.7	0.57
Chylomicron counts, venous <sup>2</sup>	60 ± 13	35 ± 7	91 ± 15	3.50
Chylomicron counts, capillary	67 ± 11	48 ± 7	105 ± 17	3.52

<sup>1</sup> F-values:  $P \leq 0.05$ , 3.55;  $P \leq 0.01$ , 6.01.<sup>2</sup> Venous total lipid and chylomicron count data include those of six underweight and six average weight subjects only.<sup>3</sup> Standard error of the mean.<sup>4</sup> Values for 7 underweight, 6 average weight and 6 overweight subjects.<sup>5</sup> Values for 6 underweight, 6 average weight and 6 overweight subjects on single samples. Chylomicron counts, particles per standard dark ground field.

# VITAMIN K ACTIVITY OF MENADIONE SODIUM BISULFITE IN CHICKENS<sup>1</sup>

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TWO FIGURES

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Experimental use of a water-soluble menadione sodium bisulfite complex in outbreaks of the chick hemorrhagic syndrome was described by Goldhaft and Wernicoff ('54) and by Frost and Spruth ('55). Although adequate controls were not used in these field studies, there appeared to be little doubt that menadione sodium bisulfite had a positive effect in reducing clotting time and mortality in the treated flocks. As cited by Goldhaft and Wernicoff, "The only product which gave any uniform results was Menadione bisulphite added to the drinking water at a level of 10 mg per gallon of water." The authenticity of these reports received independent support from Bornstein and Samberg ('54). These workers reported dramatic reduction in clotting time within 4 hours in commercial chickens in Israel following injections of menadione sodium bisulfite. Examination of these three reports strongly suggests that a marginal deficiency of vitamin K may have been aggravated by the use of sulfonamide drugs at levels and for time periods much in excess of those recommended. In line with these findings, Frost and Spruth ('55) found the requirement for vitamin K greatly increased in chicks fed a vitamin K-low diet with 0.1% of sulfaquinoxaline and discovered the vitamin K activity of menadione sodium bisulfite complex to be at least 4 times that of menadione.

<sup>1</sup> This work was reported in part to the American Institute of Nutrition in San Francisco, April, 1955. *Federation Proc.*, 14: 434.



three groups seemed inconsistent with the prevalent view that a significant rise in total serum lipids occurs following the ingestion of fat (Hetényi, '36; Peters and Van Slyke, '46; Frazer, '53). Three studies of fat tolerance following fat feeding have reported changes of total lipid concentrations of magnitudes similar to those found in this investigation (Rony and Levy, '29; Corcoran and Rabinowitch, '37; Herzstein et al., '53). Post-prandial blood lipid concentrations are known to be influenced by the amount of fat fed, by exercise, carbohydrate content of the test meal and the analysis of capillary rather than venous serum.

Following the high-carbohydrate test meal, fluctuations in serum chylomicron concentrations were observed among 4 of the overweight subjects. Although there were individual differences, the mean concentrations of the overweight group were significantly higher than those of the average weight or underweight groups at the one-half and third hours. Increases in concentrations of capillary serum total lipids also were observed following the high-carbohydrate test meal in 5 overweight subjects and two average weight subjects. Elevated serum total lipid concentrations occurred in three of the same subjects in which elevated chylomicron counts were observed, but 4 subjects showed elevated serum total lipid concentrations without measured increases in chylomicron concentrations following carbohydrate ingestion. Further, increased concentrations of the two did not necessarily occur concurrently even when both were observed in the same individual.

*Urinary findings.* Marked individual variation characterized the fasting hourly urinary nitrogen excretions; this variation existed both within groups and between days for the same individual. The differences among groups were not statistically significant. Both test meals had the effect of decreasing hourly nitrogen excretions among all groups. However, the mean total 5-hour nitrogen excretions of the underweight subjects were significantly less than those of the other two groups following both test meals. The data

TABLE 2  
Comparative activities of menadione and menadione sodium bisulfite in chicks on simplified vitamin K-low ration  
16 White Leghorn cockerels per group

ADDITION TO VITAMIN K-LOW DIET WITH 0.1% SULFAMINOXALINE	AV. 4-WEEK WT. GAIN	AV. CLOTTING TIME <sup>1</sup> minutes	NO. CHICKS > 30 MINUTES <sup>2</sup>
None	gm	minutes	
Menadione sodium bisulfite, U.S.P., 45 mg/ton	108	28 ± 2.4 <sup>3</sup>	9/10
Menadione sodium bisulfite, U.S.P., 180 mg/ton	149	> 30	14/14
Menadione sodium bisulfite, U.S.P., 180 mg/ton	151	23 ± 2.6	9/15
Menadione sodium bisulfite, U.S.P., 720 mg/ton	168	7.5 ± 1.5	0/16
Menadione, 720 mg/ton	189	27 ± 1.9	11/14
Menadione, 1440 mg/ton	161	21 ± 2.8	7/13
Menadione, 2880 mg/ton	150	12 ± 2.6	3/15
Basal diet without sulfaminoxaline	181	29 ± 1.3	15/16

<sup>1</sup>Chicks with clotting times longer than 30 minutes considered as 30 minutes in this computation. Standard error  $\sqrt{\frac{\sum d^2}{n(n-1)}}$

<sup>2</sup>Ratio of chicks with clotting time over 30 minutes to total number surviving to 4 weeks.

<sup>3</sup>Standard error.

TABLE 3  
Comparative activities of menadione and menadione sodium bisulfite in chicks on simplified vitamin K-low ration  
15 White Leghorn cockerels per group

ADDITION TO VITAMIN K-LOW DIET WITH 0.1% SULFAMINOXALINE	AV. 4-WEEK WT. GAIN	AV. CLOTTING TIME <sup>1</sup> minutes	NO. CHICKS > 30 MINUTES <sup>2</sup>	GUZZARD LESIONS <sup>3</sup>
None	gm	minutes		
Menadione sodium bisulfite, U.S.P., 0.1 gm/ton	153	> 30 ± 0.0 <sup>3</sup>	12/12	Severe
Menadione sodium bisulfite, U.S.P., 0.3 gm/ton	157	27.1 ± 2.9	11/13	....
Menadione sodium bisulfite, U.S.P., 0.3 gm/ton	136	12.3 ± 2.9	3/13	....
Menadione sodium bisulfite, U.S.P., 0.6 gm/ton	157	3.7 ± 0.3	0/15	Slight
Menadione sodium bisulfite, U.S.P., 1.0 gm/ton	169	4.2 ± 0.3	0/15	....
Menadione, 1.0 gm/ton	182	19.6 ± 2.9	6/13	....
Menadione, 2.0 gm/ton	161	22.9 ± 2.4	8/14	....
Menadione, 4.0 gm/ton	169	6.9 ± 0.7	0/12	Slight
Menadione, 6.0 gm/ton	163	7.3 ± 1.1	0/15	....
Basal diet without sulfaminoxaline	140	26.6 ± 2.4	8/10	Moderate

<sup>1</sup>Chicks with clotting times longer than 30 minutes considered as 30 minutes.

<sup>2</sup>Ratio of chicks with clotting time over 30 minutes to total number surviving to 4 weeks.

<sup>3</sup>Standard error.

among the overweight than among the average or underweight subjects; blood pyruvic acid concentrations were significantly higher among the overweight than among the underweight subjects; the ratio of blood pyruvic acid to glucose was higher among the overweight and lower among the underweight than it was among the average weight subjects; and, whereas there were no significant group differences among the various blood lipid constituents measured, fasting chylomicron counts were higher among the overweight subjects than among the other two groups.

Age has been reported as a factor influencing the fasting or basal metabolic pattern of individuals. The mean age and range of ages for subjects on the three groups in this investigation were comparable (table 1). Further, the data for the 5 subjects of 50 or more years were considered in relation to the younger subjects for all metabolites measured. In this study the rate of basal energy expenditure was not consistently decreased with age. There was no correlation of respiratory quotients, fasting glucose concentrations, fasting pyruvic acid concentrations or fasting hourly urinary nitrogen excretions with age. Although lower serum lipid concentrations occurred generally in younger subjects, the relationship between age and serum lipid concentrations was not consistent.

Interpretation of metabolic patterns following test meals is limited since individual differences may exist in both time and intensity of metabolic reactions and since statistical methods relating factors of time and degree of change are lacking. Variations of individual subjects within the overweight, underweight and average weight groups in this investigation corroborated findings of previous studies which have indicated that no single metabolic pattern is associated with the condition of overweight or underweight. Nevertheless, certain differences in metabolic patterns associated with the overweight and underweight subjects were significantly greater than variations among individuals within the groups.

TABLE 4

*Comparison of vitamin K sources in two vitamin K-low diets with and without 0.1% sulfaquinoxaline*

RATION	VITAMIN K SOURCE	AV. WEIGHT 4 WEEKS	AV. CLOTTING TIME <sup>1</sup>	PROTHROMBIN GROUP AV.	TIME % OF NORMAL
		gm	minutes	seconds	%
A.N.R.C. minus alfalfa and menadione	None	362	22 ± 2.6 <sup>2</sup> (12/18) <sup>3</sup>	177 ± 11.8	< 10
	2% alfalfa + 2 gm menadione/ton	393	2.2 ± 0.3	34 ± 0.8	67
	Menadione sod. bisulfite, U.S.P., 1 gm/ton	361	2.8 ± 0.2	29 ± 0.6	100
As above + 0.1% sulfa- quinoxaline	None	211	> 30 ( 9/9 )	...	..
	2% alfalfa	346	4.1 ± 0.5	56 ± 3.2	29
	2% alfalfa + 2 gm menadione/ton	327	3.5 ± 0.4	47 ± 1.9	37
	Menadione sod. bisulfite, U.S.P., 1 gm/ton	324	2.6 ± 0.3	35 ± 0.8	62
Simplified corn-soy	None	140	26 ± 2.3 ( 5/7 )	78 <sup>4</sup>	..
As above + 0.1% sulfa- quinoxaline	None	132	> 30 ( 5/5 )	...	..
	Menadione sod. bisulfite, U.S.P., 1 gm/ton	148	4 ± 0.9	27 ± 1.5	100

<sup>1</sup>Chicks with clotting times longer than 30 minutes considered as 30 minutes.

<sup>2</sup>Standard error.

<sup>3</sup>Ratio of chicks with clotting time over 30 minutes to total number surviving to 4 weeks.

<sup>4</sup>Prothrombin time taken on only one chick because of poor condition of group.

following the high fat test meal than those of the average or underweight subjects.

The overweight subjects had significantly higher energy expenditures at all hours following both test meals. Calorie increments above basal energy expenditures were not significantly higher than those of the other groups, however.

The underweight subjects as a group displayed a metabolic pattern which differed less from that of the average weight subjects than did the pattern of the overweight group. However, their metabolic pattern might be interpreted as indicating some greater preference for carbohydrate in metabolism. Following the high fat test meal, blood glucose concentrations showed lower elevations and decreased to fasting values more rapidly than did those of the average weight subjects; concurrently, blood pyruvic acid concentrations showed the most marked elevation above fasting values of any group and the most rapid decrease to fasting values. The resulting high pyruvic acid to glucose ratio was in contrast to the low pyruvic acid to glucose ratio at fasting among the underweight subjects. Chylomicron and total lipid concentrations early increased to the greatest extent of any group following the fat meal, but the subsequent rate of removal of these lipids from the blood was most rapid. Non-protein respiratory quotients were elevated longer than those of the other groups following the high-carbohydrate test meal.

In addition, significantly higher cumulative calorie increments following the high-fat test meal and significantly lower cumulative nitrogen excretions for 5 hours following both meals were characteristic of the underweight group.

#### SUMMARY

Metabolic patterns of a group of 7 overweight, 7 underweight and 7 average weight women were investigated at fasting and following two test meals of varying carbohydrate and fat composition. Respiratory quotients, hourly energy expenditures and hourly urinary nitrogen excretions were determined simultaneously with blood glucose, blood pyruvic

rhagic manifestations as to maintain normal prothrombin time. This is in line with the general evidence that blood clotting time is not seriously affected until prothrombin level falls below about 30% of normal. Hemorrhages were invariably found on autopsy in various tissues of birds in our tests where prothrombin concentrations were as low as 10% of normal. Occasionally hemorrhages accompanied prothrombin concentrations which were 40 to 50% of normal.

TABLE 5

*Comparative activity of menadione sodium bisulfite complex and menadione*  
10 Straight-run Production Reds per group

ADDITION TO VITAMIN K-LOW DIET <sup>1</sup>		AV. 4 WEEK WEIGHT	PROTHROMBIN	
			Average time	% of normal
		gm	seconds	
None		306	92.5 ± 10.2 <sup>2</sup>	10
Alfalfa, 2% + menadione, 2 gm/ton <sup>3</sup>		352	22.3 ± 0.6	100
Menadione sodium bisulfite complex	30 mg/ton	293	73.3 ± 6.4	14
	60 mg/ton	334	51.7 ± 3.2	23
	120 mg/ton	357	33.1 ± 1.4	49
	240 mg/ton	289	24.2 ± 1.4	77
Menadione	45 mg/ton	326	79.6 ± 7.6	13
	90 mg/ton	285	64.2 ± 3.5	18
	180 mg/ton	385	51 ± 2.3	25
	360 mg/ton	314	34.2 ± 2.1	44

<sup>1</sup> A.N.R.C. Reference ration minus alfalfa and menadione.

<sup>2</sup> Standard error.

<sup>3</sup> A.N.R.C. Reference diet.

We were interested then to use the more critical prothrombin determination in assessing the relative values of the menadione sodium bisulfite complex and menadione as sole sources of vitamin K in the modified A.N.R.C. ration. The commercial premix containing 2.52 gm menadione sodium bisulfite, U.S.P.,<sup>4</sup> per pound in calcite was used in this experiment. The experiment was set up with 10 straight-run New Hampshire (Production Red) chicks per group. The

<sup>4</sup> Four grams of Klotogen F.

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two lines shows the menadione sodium bisulfite complex to be three times as potent as menadione under these conditions.

#### DISCUSSION

The menadione sodium bisulfite complex used in these studies was found to be three times as effective as menadione as a source of vitamin K in the A.N.R.C. reference ration. This was not expected from work in the literature comparing menadione against its water-soluble forms. The classic work of Quick et al. ('54) in dogs operated upon to produce complete absence of bile from the intestinal tract showed a 5000-fold increase in requirement for vitamin K, with no increase in requirement for menadione sodium bisulfite.

The experiments in this laboratory indicate that when 0.1% of sulfaquinoxaline is added to vitamin K-low rations the requirement for vitamin K increases markedly. The requirement for vitamin K in terms of the fat-soluble menadione increases disproportionately to that of the water-soluble menadione bisulfite. This suggests that massive sulfa therapy interferes with utilization of menadione, possibly by interfering with normal production of bile.

A point which has not been generally recognized is how significantly sulfa drugs may increase the need for vitamin K. In preliminary laboratory experiments 2 gm of menadione sodium bisulfite complex per ton of feed proved inadequate to protect prothrombin levels in the presence of 0.06% of sulfaquinoxaline in the drinking water. Other experiments, to be described elsewhere, indicate that at least 10 gm of menadione sodium bisulfite complex per ton may be needed under these conditions, more than 10 times the requirement in the absence of sulfaquinoxaline. Moreover the need for menadione under similar conditions appeared to be well in excess of 40 gm per ton. Goldhaft and Wernicoff ('54) reported that 4 gm of menadione per ton did not prevent field hemorrhagic disease, whereas 10 mg of menadione bisulfite per gallon of drinking water appeared to effect a cure. Again, the field cases with which we have had direct contact (Frost and





the equally rapid recovery following vitamin K therapy. The symptoms of sulfaquinoxaline poisoning described by Delaplane and Milliff ('48) and by Davies and Kendall ('53) are not unlike those which characterize the hemorrhagic syndrome. The latter authors suggest that the clinical picture in the field is generally complicated by other disease. They cite the observation recently confirmed by Yacowitz et al. ('55) that toxic symptoms are more likely to develop in older chicks, and also cite the great variance among birds in susceptibility to the toxicity, a fact which again has a direct parallel in flocks with "hemorrhagic disease."

Research is needed under a variety of conditions of diet and disease to establish more clearly the vitamin K needs of poultry. Hill ('55) has recently indicated that resistance to fowl typhoid is increased by appropriate high levels of certain vitamins. He states, "Some vitamins, such as vitamin K, must be increased over the requirement for growth many times more than others in order to bring about increased resistance to typhoid." Fowl typhoid and many other infectious diseases of poultry lead to hemorrhagic manifestations (Goldhaft and Wernicoff, '54); again suggesting that the so-called "hemorrhagic syndrome" is not a single entity but rather a complex.

The high vitamin K potency of soybean oil was first reported by Almquist and Stokstad ('37). It is again worthy of note in the light of our findings that emergence of the "hemorrhagic syndrome" as a disease entity (Gray et al., '54; Goldhaft and Wernicoff, '54; Bornstein and Samberg, '54; and Cover et al., '55) followed the general shift from expeller to solvent extracted soybean oil meal in feeds. Attention has been called (Anderson et al., '54, '55) to the resultant decrease in vitamin K in poultry rations.

The results of the study of the effect of adding animal fat to the above rations have been quite inconsistent. In one experiment the addition of 2% of animal fat to the simplified vitamin K-low diet appeared to enhance the value of menadione. In a subsequent experiment, however, the addition

urea, urea plus methionine, linseed oil meal, subterranean clover seed, casein, and whole egg protein supplied 40% of the ration's total nitrogen and had biological values of 68.7, 75.2, 79.7, 83.0, 82.0 and 86.7 respectively for the sheep. Slen and Whiting ('55) have shown that lactalbumin is superior to linseed oil meal, peas, urea, or alfalfa for pregnant ewes. Ewes receiving lactalbumin made larger gains during pregnancy and bore heavier lambs.

With few exceptions, nitrogen balance trials with ruminants have been conducted with practical type rations containing two or more sources of nitrogen. The results from such trials cannot be attributed to any single protein but must be considered as the result of feeding a mixture of proteins.

The object of this investigation was to determine the relative efficiency of nitrogen utilization by lambs fed purified rations in which either a purified protein or urea supplied nearly 100% of the total nitrogen. Concurrent objectives were to determine metabolic and endogenous nitrogen values for lambs and to characterize each nitrogen source as to its solubility within the rumen.

#### EXPERIMENTAL

Wether lambs of Texas origin, weighing 63 to 80 lbs., were used in this experiment. A ruminal cannula was fitted in each lamb. The composition of the purified ration used in this experiment is shown in table 1. The ration was fed in two separate portions: the basal or nitrogen-free ration and the nitrogen-vitamin supplement. Preliminary tests with other lambs indicated that the ration was not palatable to most lambs over an extended feeding period. To improve palatability the basal ration was mixed with water to give it a 23% moisture content, autoclaved at 15 p.s.i. for 25 minutes, and dried over steam coils. The nitrogen and vitamin supplement was added to the basal ration at feeding. The individual nitrogen sources used were urea,<sup>2</sup> gelatin, casein, bovine blood

<sup>2</sup> Courtesy E. I. duPont Company, Wilmington, Delaware.

related to the severity of vitamin K deficiency. Growth was inhibited only when vitamin K deficiency was profound.

The incidence of the field hemorrhagic syndrome in various areas of the world since 1951-52 is thought to be related to a decreased content of vitamin K in feeds, coupled in part with overmedication with drugs which markedly increase the need for vitamin K. Hypoprothrombinemia caused by intensive sulfa medication was shown in these studies to create unusual requirements for vitamin K. High intake of a readily available source of vitamin K was found to greatly minimize the over-all injury resulting from excessive sulfa drug.

#### ADDENDUM

Recently we have observed petechial hemorrhages in commercial flocks where the blood and prothrombin clotting times were normal. These flocks showed high incidence of enteritis, slight anemia, and some abnormalities in the spleen and other tissue. It is not yet clear whether this condition results from infectious disease or dietary cause. In any case adequate vitamin K must be supplied as a prerequisite to the search for other factors which may cause hemorrhage.

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for weight, during the nitrogen-free feeding period. The nitrogen-free ration contained 0.004% of nitrogen by analysis. Lambs were force fed via ruminal cannula when feed refusals occurred on the nitrogen-free ration.

An 8-day preliminary feeding period and a 6-day collection period were used in this work. Nitrogen balance trials using the various nitrogen supplements were followed by a nitrogen-free feeding period. Fecal and urinary collections were made using metabolism stalls designed and built at this station. Total fecal and urinary output were measured daily and an aliquot of each frozen for analysis. Feces and urine were analyzed for nitrogen by the method of the Association of Official Agricultural Chemists ('45) with the exception that a 2% boric acid solution was used as the receiving fluid during distillation. Ruminal ammonia was determined by the method outlined by Hawk ('54) using Conway micro-diffusion cups.

The original experimental design was a replicated 5 by 5 Latin square with 10 randomly assigned lambs. Two sheep, one in each replicate, refused feed at sometime during the test. Therefore, the results were considered as being from randomly assigned individuals and analyzed by single classification analysis of variance as outlined by Snedecor ('46).

## RESULTS

*Endogenous and metabolic nitrogen values.* Lambs transferred to the nitrogen-free ration began refusing feed on the 5th to the 7th day following the ration change. Force feeding began when feed refusals were first noted. Two sheep scoured on the 8th day and were removed from the nitrogen-free ration. Fecal and urine collections were started on the 9th day and continued until the onset of scouring made separation of feces and urine impossible. Values obtained from the nitrogen-free period are shown in table 2.

Metabolic nitrogen was expressed two ways: milligrams per gram of dry matter intake (method 1) and milligrams per gram of fecal dry matter excreted (method 2). Metabolic ni-

REPRODUCTION AND LACTATION OF RATS  
RECEIVING CORN OIL OR BUTTERFAT  
IN THE PRESENCE OF  
SULFATHALIDINE<sup>1</sup>

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From the several studies that have been reported concerning the effects of various fats and oils on the reproductive and lactation performance of the rat it is difficult to draw any definite conclusions. Miller ('43) found that the survival rate of rats at weaning was much improved if the lard or soybean oil in the mother's diet were replaced by hydrogenated cottonseed or soybean oil. On the other hand, Loosli et al. ('44) reported that better lactation was obtained with corn oil or coconut oil than with hydrogenated coconut oil. Deuel et al. ('44) noted that corn, cottonseed, peanut, and soybean oils, and a vegetable oil margarine were just as satisfactory as butterfat in insuring successful pregnancy and lactation. In an extensive series of experiments, von Euler and his associates ('46, '47a, b) found the weaning weights of the young to be consistently higher when the mothers were fed margarine in place of butterfat.

Viswanatha et al. ('54) of this laboratory have previously reported that butterfat supported better growth in rats than

<sup>1</sup> Paper no. 3467, Scientific Journal Series, Minnesota Agricultural Experiment Station. This work has been supported by a grant from the American Dairy Association.

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used in this study might also be less abrasive to the alimentary tract than one containing wheat straw. The metabolic nitrogen level reported here is almost identical to the value (2.5 mg N per gram of dry matter intake) reported by Lofgreen and Kleiber ('53) for calves receiving a liquid diet.

The endogenous nitrogen level reported here (31.9) is only slightly lower than those reported in the literature. An endogenous nitrogen level of 33.3 has been obtained by Sotola ('30), Turk et al. ('34), and Harris and Mitchell ('41).

TABLE 3

*The true digestibilities, daily nitrogen balances, and biological values of urea, gelatin, casein, blood fibrin, and soybean protein for the lamb*

NITROGEN SOURCE	TRUE DIGESTIBILITY OF NITROGEN		NITROGEN BALANCE	BIOLOGICAL VALUE OF NITROGEN	
	% <sup>1</sup>	% <sup>2</sup>	gm N/day	<sup>1</sup>	<sup>2</sup>
Urea	78.5	82.0	0.78	51.2	53.7
Gelatin	83.9	82.2	1.66	61.2	57.4
Casein	84.6	86.5	2.16	69.2	72.7
Blood fibrin	80.5	82.5	2.78	81.6	83.1
Soybean protein	85.6	85.4	2.86	82.0	82.4
L.S.D. <sup>3</sup>					
0.05	N.S.D. <sup>4</sup>	N.S.D.	0.59	10.1	8.9
0.01	N.S.D.	N.S.D.	0.79	13.6	11.9

<sup>1</sup> Calculated from metabolic nitrogen expressed on the ingested dry matter basis.

<sup>2</sup> Calculated from metabolic nitrogen expressed on the excreted dry matter basis.

<sup>3</sup> L.S.D. least difference between means for significance.

<sup>4</sup> N.S.D. no significant difference between means.

*Nitrogen balance.* The results obtained for the different nitrogen sources are compiled in table 3. Each value in the table is the mean of 8 separate balances on 8 different lambs except for blood fibrin. Results were obtained from only 7 different lambs fed blood fibrin.

*True digestibility.* True digestibility was calculated as the percentage of nitrogen intake which was absorbed. There was no significant difference in the true digestibility of nitrogen from any of the nitrogen sources. Smaller differences between true digestibilities were obtained when metabolic nitro-

males over a period of 5 to 6 weeks were recorded as being unable to conceive. Each mother and her litter were weighed daily up to 21 days post-parturition at which time the young were weaned. Except in experiment I all litters were standardized by random selection to 6 pups on the third day following birth. Those females who possessed less than 6 young by the third day were given additional foster pups from the females who had more than 6 surviving young. The mothers were rebred following a rest period of about two weeks subsequent to the weaning of the young.

The principal criterion of the reproductive performance was the survival rate of the young within the first three days following birth (Mirone et al., '48), and the adequacy of lactation was judged primarily on the survival and weight of the young at weaning. Other observations that were employed to evaluate the reproductive and lactation performance of the females were litter size, weight of the young at birth, and the change in the weight of the mother during lactation (Vinson and Cerecedo, '44; Nelson and Evans, '47). Post mortem examinations were conducted on a number of young which had died shortly after birth, but gross inspection failed to reveal the cause of death.

## RESULTS

### *Reproduction and lactation following the first gestation period*

*Experiment I.* The female rats used in this experiment were those which had received ST-containing rations in the growth experiment previously reported (table 5, Viswanatha et al., '54). These animals were maintained on the same diet, containing either corn oil or butterfat, and were bred when they had reached maturity. Their subsequent reproductive and lactation performance is shown in table 1. All of the females in both groups successfully conceived and cast litters of comparable size and weight. By the third day, however, about 27% of the young from mothers on the corn oil diet had



pending upon the method of expressing metabolic nitrogen. When metabolic nitrogen was based on dry matter intake the values for blood fibrin and soybean protein were larger ( $P < 0.05$ ) than those for casein and gelatin which were larger ( $P < 0.05$ ) than for urea. When metabolic nitrogen was based on excreted dry matter the biological values for blood fibrin and soybean protein were larger ( $P < 0.05$ ) than that of casein which was larger ( $P < 0.01$ ) than those for gelatin or urea.

TABLE 4

*Ammonia concentrations in rumen ingesta of lambs fed nitrogen from different sources*

Values expressed as milligrams ammonia nitrogen per 100 ml of rumen ingesta

NITROGEN SOURCE	TIME AFTER FEEDING, HOURS			
	0	3	6	9
Urea	$2.0 \pm 0.3^1$	$26.0 \pm 3.3$	$12.0 \pm 4.2$	$4.6 \pm 1.2$
Gelatin	$2.8 \pm 0.7$	$4.2 \pm 1.2$	$7.0 \pm 1.7$	$8.9 \pm 2.4$
Casein	$3.8 \pm 1.3$	$6.8 \pm 2.6$	$8.1 \pm 1.6$	$3.0 \pm 0.6$
Blood fibrin	$9.4 \pm 3.9$	$3.3 \pm 1.2$	$4.6 \pm 0.8$	$5.8 \pm 1.2$
Soybean protein	$4.0 \pm 2.2$	$1.4 \pm 0.3$	$4.2 \pm 1.6$	$6.6 \pm 3.0$

<sup>1</sup> Each value is a mean of duplicate determinations on 5 different lambs.

<sup>2</sup> Standard error of the mean.

*Ruminal ammonia formation.* Ruminal ammonia concentrations exhibited by lambs fed the different nitrogen sources used in this experiment are shown in table 4.

The extent of ruminal ammonia formation varied more between animals than between nitrogen supplements. This is indicated by the large standard errors of the means in table 4. Statistically, the ruminal ammonia concentrations varied only slightly with different nitrogen sources, with the exception of urea. The ruminal ammonia concentration three hours after feeding urea was larger ( $P = 0.01$ ) than ammonia concentrations associated with any other supplement. Ruminal concentrations of 30 and 55 mg of ammonia nitrogen per 100 ml of rumen fluid three hours after feeding casein were reported by McDonald ('52) and by Annison et al. ('54).

succumbed compared to the complete survival of the offspring from the butterfat group. At this time, the mothers were allowed to nurse all their young with no restriction in the size of the litter. As judged by the weaning weights of the young, there was also a significant<sup>7</sup> difference ( $t=2.5$ ;  $P < 0.05$ ) in the lactation performance of the two groups of females in favor of butterfat.

*Experiment II.* In table 1 are also presented the results of another experiment in which the parent females had been on a stock ration<sup>8</sup> until they weighed about 120 gm. At this time the animals were divided into 4 groups: Corn oil, with and without two per cent ST, and butterfat, with and without two per cent ST. These animals were bred three weeks thereafter.

A statistical comparison of the data obtained with the two fats in the absence of ST failed to reveal any significant differences by any of the criteria listed in the first column of the table. In the presence of ST, however, the incidence of mortality of the young by the third day after parturition was 30% in the corn oil group compared to 0% in the butterfat group, thus confirming the observations of experiment I. In this experiment as well, the weights of the young at weaning were significantly higher in the litters fed butterfat ( $t=4.5$ ;  $P < 0.01$ ).

#### *Reproduction and lactation in successive gestation periods*

The females, which had successfully borne and weaned one litter on the ST-free rations in experiment II, were maintained on the same basal ration to which 2% ST had

<sup>7</sup>Significance is based on the calculation of "t" values and the corresponding levels of probability ("P") (Snedecor, '46). Only "P" values  $< 0.05$  have been considered significant.

<sup>8</sup>A mixture of 61.49% yellow corn; 10% dried skim milk; 9% each of alfalfa leaf meal, soybean oil meal and fish meal; 1.5% salt mix; and 0.01% irradiated yeast.

The biological values for casein, soybean protein, and blood fibrin are quite similar for the two species. The value of 77 for the biological value of blood fibrin for the rat is net nitrogen utilization (coefficient of true digestibility  $\times$  biological value). Assuming that 93% of the nitrogen of blood fibrin was digested by the rat, then blood fibrin has a biological value of 83 for the rat the same as reported here for the lamb. The biological values for urea and gelatin demonstrated the upgrading of poor quality ration nitrogen by rumen microorganisms and approach the value of 60 commonly reported for ruminants. Loosli et al. ('49) have reported a biological value of 56 for urea when it was fed as the sole source of nitrogen to lambs. This value is very similar to those (51.2 and 53.7) determined in this investigation. The large biological values of proteins superior to gelatin in essential amino acid distribution may indicate that the rumen flora is benefited by one or more essential amino acids. This supposition is based on reports that a large percentage (40 to 80) of the protein fed to ruminants is ultimately converted into bacterial protein (McDonald, '52 and Richardson, '55). A similar beneficial role of an amino acid for ruminants was found when methionine was added to urea rations (Williams and Moir, '51) or natural rations composed of alfalfa or field peas (Klosterman et al., '51). Regardless of the intermediate fate of essential amino acids in the rumen it seems obvious that the growing lamb, like the growing monogastric animal, responds to quality differences of proteins.

#### SUMMARY

Lambs were fed purified rations supplemented with either urea, gelatin, casein, soybean protein, or bovine blood fibrin to supply nearly 100% of the total nitrogen of the ration.

A metabolic nitrogen level of 2.39 mg of nitrogen per gram of dry matter intake or 7.17 mg of nitrogen per gram of dry matter excreted was determined for lambs fed a nitrogen-free ration.

been added. The data pertaining to the reproduction and lactation performance which followed after three successive gestation periods are summarized in table 2.

No serious interference with the ability of the females to conceive or cast litters was evident with either fat, although the ability to conceive after the second gestation period was somewhat impaired with both fats. There were also several instances of resorptions and maternal deaths at parturition; these, however, were confined to the corn oil group.

TABLE 3

*Statistical comparison<sup>1</sup> of corn oil versus butterfat with respect to weight of the young at weaning and loss in weight of the mother during lactation*  
(Based on the data shown in table 2)

LITTER SEQUENCE	WEANING WEIGHT OF YOUNG		LOSS IN WEIGHT OF MOTHER DURING LACTATION	
	t	P	t	P
First	3.0	< 0.01	2.6	< 0.05
Second	4.2	< 0.01	2.3	< 0.05
Third	2.8	< 0.05	5.3	< 0.01

<sup>1</sup> See footnote 7.

The most striking feature of these data is the sharp increase in the three-day postnatal mortality of the young after the second gestation period of the mothers receiving the corn oil diet, from a mortality of 9% in the first litter to almost 50% in the second litter. Rather unexpectedly the mortality rate in the third litter had dropped to 26%. The young from the mothers fed butterfat, on the other hand, continued to thrive through the third litter. Statistical analysis of the data pertaining to the weight of the young at weaning and the loss in weight of the mother during lactation reveals that the lactation performance of the animals of the butterfat group was also superior to that of the corn oil group in each successive litter (table 3).

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*Reproduction and lactation in successive generations*

The  $F_1$  females originating from the first litter of the parent generation shown in table 2 were allowed to mature and were then bred to produce two successive litters. The  $F_2$  females from the first of these two litters were likewise bred and studied through two gestation periods. In all cases the progeny were maintained on the same rations which had been received by their predecessors. The reproduction and lactation performance observed during the course of these studies is summarized in table 4.

With respect to the  $F_1$  generation the mortality of the young by the third day followed the same trend observed in the parent generation, namely a rather low death rate in the first litter (12%) followed by an increase (42%) in the second litter. The  $F_1$  females on butterfat again produced litters which survived the first three critical days of life in both litters. In contrast to the parent generation, however, no significant difference in lactation, as judged by the weight of the young at weaning or by the loss in weight of the mothers during the nursing period, was apparent between the two fats.

A rather surprising result was the almost complete absence of infantile mortality in both litters produced by the  $F_2$  females on the corn oil diet. This was in sharp contrast to the poor survival rate in litters of the parent and  $F_1$  females on the same ration. As in the case of the  $F_1$  females, no difference in lactation performance between the mothers on the corn oil and butterfat diets was revealed.

## DISCUSSION

Since the mortality of the young was the principal point of difference between the corn oil and butterfat diets, it is important to point out that the mortality figures have been based on the *total* number of young which failed to survive in any one group. This, of course, gives no indication of the variations in the mortality rate of individual litters within



present in butterfat but absent in corn oil, is suppressed. The capacity of certain microorganisms to develop a resistance to sulfa drugs is not unknown (Sevag and Green, '44), and it is conceivable that the continued ingestion of ST over a period of several generations may have led to the gradual establishment of a ST-resistant microflora. The improvement in the lactation performance of the  $F_1$  and  $F_2$  females fed corn oil over the preceeding parent generation may also be a manifestation of this adaptation phenomenon on the part of the intestinal flora. The fact that the  $F_1$  females on corn oil reproduced so poorly and yet managed to lactate as satisfactorily as their butterfat counterparts indicates that the nutritional requirements for a normal reproduction may be more stringent than those required for lactation, a view previously expressed by Sica and Cerecedo ('48).

An alternative explanation which does not invoke the concept of an unrecognized nutrient in butterfat is suggested by the recent paper of Thomasson ('55) relating to the possible presence of growth-inhibiting substances in certain vegetable oils. In order to explain the observations recorded here on this basis, it becomes necessary to postulate that ST must in some way potentiate the effects of the harmful substances present in corn oil. The manner in which ST would exert this effect is not readily apparent.

#### SUMMARY

Corn oil and butterfat in the presence of 2% sulfathalidine have been compared with respect to their ability to promote reproduction and lactation of female rats in successive litters and generations.

In studies on the parent generation, the postnatal mortality of the young from mothers on the corn oil diet ranged from 9 to 48% within the first three days, tending to be highest after the second gestation period. When corn oil was replaced with butterfat almost complete survival of the young was obtained. Lactation performance, as judged by the weight of



Twenty-one weanling rats of the Wistar strain were de-salivated by the block dissection method in which the ducts to the major salivary glands are ligated and cut and the glands excised. This method is described in detail elsewhere (Haldi, Wynn, Shaw and Sognnaes, '53). The animals were divided into three groups. Two groups were fed diets that have been designated in a previous publication (Wynn, Haldi, Shaw and Sognnaes, '53) as the Emory and the Harvard diets. The basic composition of these diets is given in table 1. The former has been found to be slightly cariogenic whereas the latter produces severe caries in a relatively short time.

TABLE 1

*Composition of the Emory and Harvard high sucrose diets<sup>1</sup>*

CONSTITUENTS	EMORY DIET	HARVARD DIET
	%	%
Sucrose	64	64
Casein	20	23
Hydrogenated vegetable oil	8	..
Corn oil	..	5
Yeast and liver extract	4	..
Liver concentrate	..	4
Salt mixture <sup>2</sup>	4	4

<sup>1</sup> Vitamin supplements were added to each of these diets. For composition see Wynn et al. ('53).

<sup>2</sup> The composition of the salt mixture is given in Wynn et al. ('53).

The third group was fed a commercial laboratory chow<sup>2</sup> in order to have, for comparative purposes, animals with little or no caries. We had found in previous experiments that this stock diet is practically non-cariogenic.

When the animals had been on the experiment 70 days, at which time no caries had developed in the rats on the stock diet, only a moderate amount of caries on the Emory diet, and extensive caries on the Harvard diet, pH readings were obtained on all the animals on the three diets 4 different times during the day. Eight readings (two in each quadrant) were taken each time. This gave a total of 224 readings on

<sup>2</sup> Purina.

- SURE, B. 1941 Dietary requirements for fertility and lactation. XXIX. The existence of a new dietary factor essential for lactation. *J. Nutrition*, 22: 499.
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The caries score ranged from 1 to 5 depending on the extent of the lesion. All adherent tissue was then carefully removed, the roots cut off and each tooth dropped into a separate vial containing 10 ml of 1%  $\text{CuSO}_4$  solution, preparatory for the determination of lactate. The procedure for lactate analysis was that described by Barker and Summerson ('41) as modified by Moore ('52) for determining such extremely small amounts of lactate as are present in the rat's tooth. According to Barker and Summerson ('41) this method is highly specific for lactate in biological materials. This specificity was further demonstrated by Moore ('52). We found this method very satisfactory. In a preliminary lactate determination on the noncarious teeth of an animal selected at random, there was an average difference of only  $0.3 \mu\text{g}$  between the lactate on the first molars in the right quadrant and on the corresponding teeth in the left quadrant.

## RESULTS

*Experiment 1.* A striking feature of the 224 pH readings taken on each group of animals the day they were sacrificed was the large number (82%) of the readings above 7.0 on the teeth of the animals on the stock diet and a progressively smaller number of these higher readings (35 and 22% respectively) on those of rats fed the Emory and Harvard diets. On the other hand, there were no readings below pH 6 on the teeth of rats fed the stock diet, whereas there were 3 and 25% below pH 6.0 on the Emory diet and Harvard diet, respectively. The distribution of pH readings is shown in the nomogram in figure 1. Examination of the teeth revealed, as had been anticipated, that there was no caries in the teeth of the animals on the stock diet, whereas on the Emory diet there was a moderate amount of caries with the score ranging from 0 to 2 and on the Harvard diet extensive caries with a score of 1 to 5.

The relationship between the pH readings on the teeth and the caries score is shown in figure 2. It will be noted that there was only a slight drop in the average pH as the caries

# RESPONSES OF RATS TO UREA AND RELATED SUBSTANCES<sup>1</sup>

## THE USE OF A SPACED-FEEDING TECHNIQUE

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The practice of feeding rats for limited portions of the day has been investigated by Werthessen ('37) and Barker ('49), and has been used in studies of the respiratory quotient (Tepperman et al., '43) and of fatty acid synthesis (Dickerson et al., '43). More recently Lepkovsky et al. ('55a,b) have employed spaced feeding in investigations of glycogen synthesis. We have used this procedure in proteinuria studies in an attempt to reduce the contamination of urine by spilled food (Rumsfeld and Baumann, '55; Finlayson and Baumann, '56). In the latter studies 12.5% of urea in the diet depressed growth severely in rats fed two hours a day, but when the rats were regrouped and the urea diet was fed ad libitum, no depression occurred. The present experiments deal with the toxicity of urea when fed two hours per day and include attempts to use spaced feeding for determining the biological values of proteins and for altering the effects of mildly toxic compounds.

## METHODS

Holtzman male albino rats were used in all series. They were housed in individual wire-bottom cages and were given

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<sup>2</sup>Public Health research fellow.

were obtained from the teeth of the animals that had been fed the Harvard diet as it was only this group of animals that had carious lesions with scores greater than 2. The pH readings, as stated previously, were taken on the day the animals were sacrificed. Four readings were made on each tooth at different times of the day and the average of these 4 readings taken as the representative pH reading for the tooth.

*Experiment 2.* The results obtained in the second experiment are presented in table 2. The relationship between the caries score and the pH on the surface of the teeth was the same as in the first experiment. In both experiments there

TABLE 2

*pH, lactate and caries scores on the teeth of rats fed the Harvard diet for varying lengths of time<sup>1</sup>*

Caries score	0	1	2	3	4	5
Average pH	6.3 $\pm$ 0.4	6.1 $\pm$ 0.4	6.2 $\pm$ 0.2	6.1 $\pm$ 0.3	5.6 $\pm$ 0.2	5.3 $\pm$ 0.3
Readings below 6.0, %	16	25	33	67	83	100
Total lactate, $\mu$ g	10 $\pm$ 3	13 $\pm$ 2	14 $\pm$ 4	15 $\pm$ 6	38 $\pm$ 11	50 $\pm$ 15

<sup>1</sup> Values in the table were obtained on the first and second upper and lower molars of 18 rats (144 teeth) with varying degrees of caries.

was very little change in the pH as the score rose from 0 through 3 whereas an appreciable drop occurred with progression of the caries score from 3 through 4 and 5. Likewise, with an increase in the caries score from 0 to 2, there was a very moderate increase (from 16 to 33%) in the number of readings below 6.0. When the carious lesions had advanced to the point where they gave a score of 4, 83% of the pH readings were below 6.0. With further spread of the lesion, 100% of the readings were below this level.

A direct relationship was observed between the caries score and the amount of lactate recovered from the tooth. As shown in table 2, there was very little difference in the pH and the lactate on the teeth with a caries score of 1 through 3;

depression was usually not apparent until the level of dietary urea exceeded 25%. Moreover, resistance to urea on either regime was not altered by the nutritional adequacy (presence or absence of added cystine) or the level (12 or 20%) of the protein in the diet (Finlayson, '55).

To equate the two methods of feeding, rats received 0, 5, or 10% of urea for two hours per day or 0, 20, or 30% of

TABLE 1  
*Growth effects of urea fed ad libitum or two hours per day*

FEEDING METHOD	UREA <sup>1</sup>	5 WK. GAIN	% DEPRESSION <sup>2</sup>		DAILY FOOD INTAKE	UREA INTAKE	
			5 wk.	Average <sup>3</sup>			
	%	gm			gm	gm/day	gm/hr. <sup>4</sup>
Spaced	0	36 ± 9 <sup>5</sup>	0	0	8	0	0
	5	24 ± 9	33	38	8	0.4	0.2
	10	7 ± 4	81	85	7	0.7	0.35
Ad libitum	0	148 ± 12	0	0	17	0	0
	(20) <sup>6</sup>	144 ± 9	3	3	22	0	0
	(30) <sup>6</sup>	141 ± 10	5	7	23	0	0
	20	128 ± 12	14	21	18	3.6	0.15
	30	93 ± 6	37	39	16	4.8	0.2

<sup>1</sup> Added at expense of entire diet.

<sup>2</sup>  $\frac{\text{Gm depression from controls}}{\text{Gm gain of controls}} \times 100.$

<sup>3</sup> Average of weeks 2-5.

<sup>4</sup> Daily urea intake divided by hours food was available per day.

<sup>5</sup> Standard error of 5 rats.

<sup>6</sup> Values in parentheses are cellulose (Solka Floc) levels.

urea ad libitum. Growth was depressed progressively as the level of dietary urea was increased, the percentage of urea required to produce a given depression being much lower when the feeding was spaced (table 1). Although 5% of urea, using the spaced-feeding technique, depressed growth as much as did 30% ad libitum, the daily urea intake of rats on the latter regime exceeded the former by a factor of 12. Growth depression appeared to be determined by the rate at which urea was ingested: rats in each of these groups

## SUMMARY AND CONCLUSIONS

In one experiment three groups of weanling albino rats were desalivated and fed from weaning diets that were non-cariogenic, moderately and severely cariogenic. Determinations of the pH on the tooth surfaces were made at the end of 70 days, the animals sacrificed and teeth scored for caries.

In the animals with no caries there were no pH readings below 6.0. As the caries score advanced from 1 to 3, there was a slight but insignificant drop in the average pH. The drop in pH was much more pronounced and was statistically significant as the caries score went from 3 through 4 to 5.

In another experiment desalivated albino rats of the same colony as in the previous experiment, were fed a highly cariogenic diet from weaning. Two animals were sacrificed after two weeks on the diet and then two more each successive week. Readings of the pH on the molar teeth were taken immediately before sacrifice. The teeth were then scored for caries and analyzed for lactate.

A direct relationship was observed between the pH, the amount of lactate recovered from the tooth and the caries score. Pronounced changes in pH and lactate on the tooth were obtained only when there was severe caries.

It is apparent that the intrinsic processes involved in the spread of carious lesions in rats' teeth in these experiments were associated with a lowering of pH and an increase in the lactate in and on the teeth.

## ACKNOWLEDGMENT

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TABLE 3

*Effect of spaced feeding on the toxicity of various compounds*

DIETARY ADDITION	5-WEEK GAIN WITH STANDARD ERROR						PER CENT DEPRESSION <sup>1</sup>			
	Ad libitum			Spaced			Ad libitum		Spaced	
	Series A		Series B	Series A		Series B	Series A		Series B	
	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
None	169 ± 2	177 ± 8		72 ± 9	74 ± 9		0	0		0
Glycine <sup>2</sup>	75 ± 14			46 ± 6			55	36		
2.5% L-Leucine	134 ± 7	144 ± 10		19 ± 5	38 ± 5		21	19		49
10% Diammonium citrate	123 ± 4			32 ± 4			27	56		
5% Ammonium carbonate	124 ± 20	171 ± 6		12 ± 12	37 ± 13		27	3		50
20% Ethanol in drinking water	131 ± 5			68 ± 9			22	6		
0.05% 3'-Methyl-4-dimethylaminobenzene		74 ± 8 (4/5) <sup>3</sup>			48 ± 5			58		35
0.35% 2,4-Dinitrotoluene		56 ± 8			12 ± 9 (3/5) <sup>3</sup>			68		84
10 ppm Selenium <sup>4</sup>		... (0/5) <sup>3</sup>			8 ± 4			(96) <sup>5</sup>		89

<sup>1</sup> Gm depression from controls  

$$\frac{\text{Gm gain of controls} - \text{Gm gain of treated}}{\text{Gm gain of controls}} \times 100.$$
<sup>2</sup> Eight per cent of ad libitum ration; 5% of spaced; all compounds added at expense of the entire diet.<sup>3</sup> Figures in parentheses indicate survival when less than 100%.<sup>4</sup> Added as Na<sub>2</sub>SeO<sub>3</sub>.<sup>5</sup> Reflects gain at three weeks, when all rats were alive.





TABLE 4

*Blood urea and ammonia levels after eating ammonium salts<sup>1</sup>*

DIETARY ADDITION	BLOOD AMMONIA NITROGEN				BLOOD UREA NITROGEN			
	Ad libitum		Spaced		Ad libitum		Spaced	
	Series A	Series B	Series A	Series B	Series A	Series B	Series A	Series B
None	mg % $2.2 \pm 0.2^2$	mg % $1.9 \pm 0.1$	mg % $< 2.4^3$	mg % $2.0 \pm 0.2$	mg % $9.6 \pm 1.8$	mg % $9.3 \pm 0.8$	mg % $9.9 \pm 1.9$	mg % $7.3 \pm 1.9$
5% Ammonium carbonate	$2.0 \pm 0.1$	$2.0 \pm 0.3$	$< 2.4$	$1.9 \pm 0.1$	$10.6 \pm 1.9$	$7.8 \pm 1.5$	$35.3 \pm 4.1$	$25.9 \pm 3.8$
10% Diammonium citrate	$2.8 \pm 0.6$		$< 2.4$		$9.1 \pm 2.9$		$26.4 \pm 3.5$	

<sup>1</sup> Blood withdrawn from "spaced-fed" rats two hours after eating.<sup>2</sup> Standard deviation.<sup>3</sup> Samples available were too small for precise determinations.

logical values were employed and alcaptonuria was produced by supplementing these diets with either phenylalanine or tyrosine. A comparison of the effectiveness of the DL and L forms of these aromatic amino acids in producing alcaptonuria was also made.

#### EXPERIMENTAL

*Care of the animals.* Male white rats of approximately 150 gm weight from litter groups of 4 were kept in separate metabolism cages. They were given a diet of bread, milk and lettuce for three to 4 days prior to the administration of the synthetic diet. After a control period of 6 to 8 days the animals were fed extra doses of the aromatic amino acid for three successive days. Four to 6 days after the last feeding of the aromatic amino acid, the urinary excretion of HA had again reached that of the control level. Hence it took about 15 days to complete one experiment. The rats were weighed weekly, and also before and after each dosage period.

*Urine collection.* Urine collection was started three to 4 days after the synthetic diet was given. Twenty-four-hour urine samples were collected in the presence of 1.5 ml of glacial acetic acid as the preservative. The urine and the washings were combined, filtered and diluted to 100 ml. Two milliliters of the diluted alcaptonuric urine samples or 10 ml of the diluted control urine samples was used for the analysis of HA.

*Diets.* The synthetic diets used varied only in the kind and amount of the protein used. The general composition was as follows: vitamin mixture 1%, salt mixture (Osborne and Mendel, '19) 4%, sucrose 24%, corn oil 4%, cod liver oil 2% and protein plus starch 65%. The vitamin mixture contained per 98 gm starch: 37 mg thiamine, 37 mg pyridoxine, 75 mg riboflavin, 300 mg calcium pantothenate, 300 mg *p*-aminobenzoic acid and 375 mg nicotinic acid. The protein of the diet was increased at the expense of starch. The protein and amino acid supplements of the diets are listed in the first columns of tables 1 and 2.

lett et al. ('16) that to produce toxic effects, a "large quantity of urea must be taken within a brief period of time."

Toxicities of L-leucine and dinitrotoluene were also increased by spaced feeding. Growth retardation by leucine is apparently caused by an isoleucine antagonism (Harper et al., '55), but the reasons for an enhancement of this effect by spaced feeding are obscure. In rats fed dinitrotoluene, those which failed to survive had eaten relatively little, suggesting that the apparent increased toxicity may have been complicated by starvation, as previously observed in mice by Clayton and Baumann ('44; '48). The use of spaced feeding for determining the biological values of proteins appears to have several advantages despite the care necessary in training the animals. The protein under study need be fed for only two weeks which, on a two hour per day feeding regime, would require only a small amount of the test material.

#### SUMMARY

Spaced feeding, the practice of feeding rats for only two hours per day, has been found to increase the growth-depressing action of several nitrogenous compounds. Dietary urea depressed growth in both spaced and orthodox experiments; 5% of urea fed two hours per day was as effective as 30% fed ad libitum. The depression in growth has been correlated with the rate of urea intake and the level of urea in the blood, and was not affected by the level or adequacy of the dietary protein.

Spaced feeding increased the toxicity of L-leucine, diammonium citrate, ammonium carbonate, and 2,4-dinitrotoluene. Growth depressions by the ammonium salts varied directly with blood urea. This regimen lessened the toxicity of 3'-methyl-4-dimethylaminoazobenzene and ethanol but had little effect on relative growth rates when biotin, vitamin B<sub>12</sub> and folic acid were omitted from the diet or when glycine or antibiotics were added. The procedure shows promise in measuring the biological value of small amounts of protein.

*Chemical methods.* Ammoniacal silver nitrate, freshly prepared each day, was used at room temperature as a qualitative test for the onset of alcaptonuria. The iodometric titration method as described by Neuberger ('47) was used for the quantitative estimation of HA. The method was standardized with a sample of synthetic HA generously supplied by Dr. Lynn D. Abbott Jr.

TABLE 2

*The effect of the administration of L and DL forms of tyrosine and phenylalanine on the excretion of homogentisic acid (HA)*

DIET	NO. OF RATS	ISOMER	AMINO ACID FED PER 100 gm BODY WEIGHT	EXTRA HA EXCRETED PER gm AMINO ACID FED
			gm <i>Phenylalanine</i>	mg
Casein, 5%	2	L <sup>1</sup>	1.10 ± 0.19 <sup>2</sup>	0.46 ± 0.12
Casein, 5%	6	DL <sup>3</sup>	1.03 ± 0.04	4.09 ± 0.42
Casein, 20%	5	L	1.00 ± 0.11	0.22 ± 0.11
Casein, 20%	10	DL	1.04 ± 0.05	2.84 ± 0.03
Casein, 50%	8	L	0.86 ± 0.11	0.03 ± 0.01
Casein, 50%	6	DL	0.79 ± 0.08	3.51 ± 1.00
			gm <i>Tyrosine</i>	mg
Casein, 20%	13	L <sup>4</sup>	1.31 ± 0.08	17.60 ± 8.36
Casein, 20%	11	DL <sup>4</sup>	1.41 ± 0.06	0.09 ± 0.03
Zein, 21.2%	6	L	1.08 ± 0.20	140.7 ± 43.3
Zein, 21.2%	7	DL	1.41 ± 0.30	2.78 ± 1.18

<sup>1</sup> This amino acid was supplied to Professor Lewis by Dr. Seiichi Izume. A description of this preparation appeared in footnote 2 of Chandler and Lewis ('32).

<sup>2</sup> The mean and its standard error.

<sup>3</sup> Winthrop-Stearns Incorporated.

<sup>4</sup> Merck and Company.

## RESULTS

*Effect of the nature of the dietary protein on the production of alcaptonuria.* Table 1 summarizes the effect of various diets on the excretion of HA. It is noted in this table that the increased excretion of HA was 15 times as great in rats receiving the 18.3% gliadin diet as in their pair-fed litter mates on the 18.4% casein diet. However, administra-

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diet. In the presence of added DL-phenylalanine in the diet, a deficiency in both lysine and tryptophan has thus induced a much greater increase in the excretion of HA than a deficiency in lysine alone. It may also be pointed out that while rats on the gliadin plus lysine diet excreted no greater amount of HA than their casein-fed paired mates, rats on the zein plus lysine and tryptophan diet excreted considerably more HA than their casein-fed controls.

*Effects of the administration of L and DL forms of phenylalanine and tyrosine on the excretion of HA.* In a preliminary experiment, 8 rats were fed a 20% casein diet for three days. The average daily excretion of HA on this diet was found to be 0.77 mg with a range from 0.62 to 0.91 mg per rat. Under similar conditions, the average daily output of HA was 0.74 (0.68 to 0.79) mg and 0.64 (0.57 to 0.70) mg per rat respectively on 50 and 5% casein diets. These results show that varying the casein content of the diet over a range of 5 to 50% did not affect the level of HA excretion. Furthermore, it can be seen in table 2 that the difference in the excretion of extra HA following the addition of L- or DL-phenylalanine to these three casein diets was very slight indeed.

When the effectiveness of L- and DL-phenylalanine in producing HA was compared, it was found that DL-phenylalanine always caused a greater increase in the HA excretion than did the same amount of the L isomer. On the other hand, administration of L-tyrosine to rats given the 20% casein diet resulted in remarkably higher excretion of HA than that of the DL isomer. The increase in the excretion of HA was greatly enhanced by substituting the casein diet with the zein diet. Again, L-tyrosine was found to be much more effective than DL-tyrosine. The extraordinary behavior of L-tyrosine in this respect deserves attention. It is interesting to point out that on the 20% casein diet, the aromatic amino acids, in the order of decreasing effectiveness in increasing the HA level of the rat urine, are L-tyrosine, DL-phenylalanine, L-phenylalanine, and DL-tyrosine.

# QUANTITATIVE RELATIONSHIPS OF TRYPTOPHAN AND NICOTINIC ACID IN THE BABY PIG<sup>1</sup>

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## ONE FIGURE

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Many reports of a qualitative nature have succeeded that of Krehl et al. ('45), in which it was shown that tryptophan would promote growth in nicotinic acid-deficient rats (Woolley, '46; Krehl et al., '46a; Henderson et al., '47; Rosen et al., '46). Nicotinic acid in the diet has been shown to decrease the requirement of the rat for tryptophan (Krehl et al., '46b). Salmon ('54) has shown that the tryptophan requirement of the rat is enhanced with increases in dietary protein or by the omission of nicotinic acid. Studies with the chick have demonstrated an L-tryptophan requirement of 0.15% and a nicotinic acid requirement of 10 mg%. In the absence of nicotinic acid the L-tryptophan increased to 0.20% of the diet (Fisher, '54). The first study of the requirements of the pig for nicotinic acid (Hughes, '43), set the requirement at 5 to 10 mg/100 lbs. body weight or 0.11 to 0.22 mg/kg body weight per day. Subsequent work (Powick et al., '47), using younger pigs on high protein diets raised the estimated requirement to 0.6 to 1.0 mg/kg body weight per day. High protein diets and consequently high levels of tryptophan have been shown to completely satisfy the nicotinic acid requirement (Powick et al., '48; Cartwright et al., '48). Age and

<sup>1</sup> The data reported in this paper are taken from a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.



has been found (Berg and Bauguess, '32; Chase and Lewis, '34; Dakin, '08; Wilson and Lewis, '29). However, there is evidence to indicate that D-tyrosine when ingested in the DL form is totally unavailable for normal use in man (Albanese et al., '46). By employing Millon's reaction (Folin and Ciocalteu, '27) as modified by Medes ('32), a much higher "tyrosine" value was found in the urine of rats fed DL-tyrosine than in that of those fed L-tyrosine. It may be assumed that a part of the DL-tyrosine fed is excreted in the urine and what remains in the body is insufficient to give rise to extra HA in a well-fed animal. It is also possible that the toxic effect of L-tyrosine (Martin, '42-'43; Schweizer, '47) may impair the normal ability of the body to metabolize large quantities of the aromatic amino acids, and consequently it favors the production of experimental alcaptonuria.

#### SUMMARY

Deficiency in lysine and in both lysine and tryptophan very greatly enhanced the homogentisic acid excretion following the ingestion of DL-phenylalanine. This effect was completely abolished by supplementing the gliadin diet with lysine, but only partially abolished by supplementing the zein diet with lysine and tryptophan.

Administration of L-phenylalanine to rats fed casein diets caused lower excretion of homogentisic acid than the same dose of the DL isomer. DL-Tyrosine, on the contrary, did not increase the urinary excretion of homogentisic acid in the casein-fed rats, whereas the same amount of the L-isomer produced a considerable output of homogentisic acid. When the zein diet was used, DL-tyrosine also caused an increased excretion of homogentisic acid, but L-tyrosine brought about very severe alcaptonuria.

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DL-tryptophan requirement when there was no nicotinic acid in the diet.

# EXPERIMENTAL

Two- to three-day-old baby pigs were obtained from a commercial source for use in these experiments. They were housed individually in wire bottom metal cages and fed ad libitum a hydrolyzed casein synthetic milk assayed to be nicotinic acid and tryptophan free. The composition of the basal diet is given in table 1. The feeding and care of the animals was similar to that described by Johnson et al. ('48). All experiments were of 4 weeks duration which was sufficient time for

TABLE 2

*Effect of 7 levels of DL-tryptophan on growth and feed efficiency in baby pigs<sup>1,2</sup>*

ITEM	% DL-TRYPTOPHAN						
	0.1	0.125	0.15	0.175	0.20	0.25	0.30
Av. initial wt., kg	2.33	2.37	2.48	2.47	2.50	2.30	2.28
Av. final wt., kg	3.13	3.37	4.42	6.02	6.28	7.73	8.68
Av. gain, kg	0.80	1.00	1.93	3.55	3.78	5.42	6.40
Feed consumed, kg	3.61	3.47	4.60	5.83	6.13	7.69	8.21
Feed/gain, kg	4.51	3.47	2.38	1.65	1.62	1.42	1.28

<sup>1</sup> Nicotinic acid was added to the diet at 50 mg per kilogram of dry matter.

<sup>2</sup> Three pigs in each group.

wide differences to occur between treatments in all tests. Differences usually became apparent at 10 to 14 days and widened through the remainder of the test period.

The individual experiments are given in tables 1 through 7.

# RESULTS

## *DL-tryptophan requirement in presence of excess nicotinic acid*

*Experiment 1.* The data from experiment 1, as shown in table 2, indicated that the DL-tryptophan requirement in the presence of an excess of nicotinic acid was higher than had been anticipated.

- PAPAGEORGE, E., AND H. B. LEWIS 1938 Comparative studies of the metabolism of the amino acids. VII. Experimental alcaptonuria in the white rat. *Ibid.*, 123: 211.
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*Nicotinic acid requirement in presence of required level of DL-tryptophan*

*Experiment 3.* Results of this experiment are summarized in table 4. Both growth and feed efficiency reached a maximum at the 17 mg/kg level. The gains (fig. 1 b) are best described by a straight line fitted through all of the points indicating that a plateau may not have been reached. However, the feed required per unit of gain, as shown in table 4, had reached a minimum at the 17 and 20 mg/kg levels.

In view of the lack of groups receiving higher levels of nicotinic acid supplementation the requirement has not been

TABLE 4  
*Nicotinic acid requirement of the baby pig<sup>1,2</sup>*

ITEM	NICOTINIC ACID (MG/KG DRY MATTER)					
	5	8	11	14	17	20
Av. initial wt., kg	1.79	1.85	1.83	1.81	1.80	1.75
Av. final wt., kg	4.00	4.62	4.05	5.38	6.09	5.58
Av. gain, kg	2.21	2.77	2.23	3.56	4.29	3.83
Av. feed consumed, kg	4.25	4.35	4.46	4.93	5.46	4.91
Feed/gain, kg	1.92	1.57	2.00	1.38	1.27	1.28

<sup>1</sup> DL-Tryptophan was added to the diet at 0.3%, the level indicated as required in experiments 1 and 2.

<sup>2</sup> Four pigs in each group.

conclusively demonstrated. However, on the basis of the feed required per unit of gain and the lack of increase in gain at the level of 20 mg/kg it seems probable that the requirement is not over 20 mg/kg of diet when the diet contains the minimum amount of tryptophan necessary for normal growth (0.3% of DL-tryptophan).

*L-tryptophan requirement in the presence of excess nicotinic acid*

*Experiment 4.* Gains reached a plateau at the 0.2% L-tryptophan level, which represents the approximate requirement

Recently in this laboratory the effect of the thyroid on the formation of vitamin A from carotene, administered intravenously as an aqueous dispersion in Tween 40<sup>1</sup> (polyoxyethylene sorbitan monopalmitate), has been studied in rats. Parenteral administration was chosen in preference to the oral route in order to overcome any possible effects that variations in absorption might have on the results. Carotene was administered intravenously as an aqueous dispersion since it is now well recognized that under these conditions it is readily converted to vitamin A (Bieri and Pollard, '54; Kon, McGillivray and Thompson, '55; McGillivray, Thompson and Worker, '56), whereas when injected as an oily solution it appears to be inactive (Sexton, Mehl and Deuel, '46). In preliminary experiments (McGillivray et al., '56), using blood levels of vitamin A and liver storage as criteria, the thyroid was shown to be without effect on the conversion of carotene to vitamin A. The present paper covers subsequent work of a similar nature using blood levels and liver storage of vitamin A, and in addition the remission of xerophthalmia, as criteria.

#### EXPERIMENTAL

All rats used in these experiments were albinos of the Wistar strain inbred from stock introduced into New Zealand by Dr. I. J. Cunningham, Superintendent of the Wallaceville Research Station, Wellington. Those used in experiment I were maintained throughout life on a basal diet<sup>2</sup> of the following percentage composition: ground wheat, 42; ground barley, 10.5; ground oats, 4; dried skim milk, 34; wheat germ, 8; CaCO<sub>3</sub>, 1; NaCl, 0.5. At slaughter (200 to 300 gm) they were almost deficient in vitamin A as evidenced by low levels of vitamin A in the blood and the absence of appreciable quantities in the liver. The rats used in experiment II were maintained on the U.S.P., vitamin A-free test diet (Hawk, Oser and Summerson, '47) from weaning until such time as xerophthalmia became apparent, a period usually of 4 to 6 weeks.

<sup>1</sup> Atlas Powder Company, Wilmington, Delaware.

<sup>2</sup> Prepared in pellet form by W. and R. Fletcher, Ltd., Wellington, New Zealand.

received approximately 1.3 mg of nicotinic acid. Subsequent supplementation with nicotinic acid was not necessary during the course of the experiment. Apparently the 2- to 3-day old pig is unable to convert tryptophan to nicotinic acid and has a very low reserve of this vitamin.

TABLE 6

*The DL-tryptophan requirement of the baby pig in the absence of nicotinic acid (Experiment 5)<sup>1</sup>*

ITEM	% DL-TRYPTOPHAN				
	0.3	0.35	0.4	0.45	0.5
No. pigs	2	3	4	2	3
Av. initial wt., kg	1.25	1.33	1.39	1.35	1.30
Av. final wt., kg	4.05	4.39	5.35	4.70	4.00
Av. gain, kg	2.85	3.30	3.96	3.35	2.70
Av. feed consumed, kg	5.83	6.00	6.71	6.27	5.97
Feed/gain, kg	2.05	1.82	1.69	1.87	2.21

<sup>1</sup> A different source of hydrolyzed casein which was not entirely salt-free was used and is the cause of the lower gains in this experiment.

TABLE 7

*The DL-tryptophan requirement of the baby pig in the absence of nicotinic acid (Experiment 6)*

ITEM	% DL-TRYPTOPHAN						
	0.3 + NA <sup>1</sup>	0.3	0.35	0.4	0.45	0.5	0.6
No. pigs	3	1	3	3	3	3	2
Av. initial wt., kg	1.35	1.47	1.37	1.40	1.40	1.43	1.35
Av. final wt., kg	4.62	3.50	3.88	4.83	5.70	5.82	4.90
Av. gain, kg	3.27	2.03	2.51	3.43	4.30	4.39	3.55
Av. feed consumed, kg	4.63	3.36	4.19	4.75	5.25	5.23	4.93
Feed/gain, kg	1.42	1.65	1.67	1.38	1.22	1.19	1.39

<sup>1</sup> Group 1 pigs received in addition to 0.3% DL-tryptophan 50 mg of nicotinic acid per kilogram of dry matter in the diet.

Growth was erratic and suboptimum throughout the experiment due to the excess salt in the hydrolyzed casein. The pigs scoured continually and 6 died during the 4 weeks. The results are summarized in table 6.

It is evident from table 6 that growth was below normal and that feed efficiencies were suboptimal. However, these

TABLE 1

## Experiment I

*Effect of thyroid activity on the conversion of carotene to vitamin A in rats partially deficient in the vitamin. Vitamin A content of plasma and liver 24 hours after the administration of an aqueous dispersion of carotene in Tween 40*

RATS		TREATMENT	OXYGEN CONSUMP- TION	CARO- TENE ADMIN- ISTERED	BLOOD PLASMA		LIVER			
No. used	Mean weight				gm	Vita- min A alcohol	CARO- TENE $\mu$ g/100 ml	Vitamin A		CAROTENE
								Alcohol	Ester	
2	230	Sham operated 28 days prior to injections	litres/ kg/hour 35	$\mu$ g None	$\mu$ g/ 100 ml 11	$\mu$ g/ 100 ml 0	$\mu$ g 0.5	$\mu$ g 0.6	$\mu$ g 1.1	Trace
3	230	Sham operated 28 days prior to injection	35	400	19	Trace	4.4	6.6	11.0	15
5	200	Sham operated; 30 $\mu$ g 1-thyroxine subcutaneously daily for 28 days prior to injection	52	400	17	Trace	5.0	5.6	10.6	11
5	300	Sham operated; 250 mg thiouracil daily in food for 28 days prior to injection	20	400	21	Trace	5.2	7.6	12.8	17
5	300	Thyroidectomised; 100 mg thiouracil daily in food for 28 days prior to injection	23	400	18	Trace	3.8	6.4	10.2	14

TABLE 2

## Experiment II

*Effect of the thyroid on the remission of xerophthalmia and on weight increases in rats following intravenous injection of carotene as an aqueous dispersion in Tween 40*

NO. RATS USED	MEAN WEIGHT gm	TREATMENT	CAROTENE ADMINISTERED $\mu$ g	INTERVAL OVER WHICH OCULAR SYMPTOMS DIS- APPEARED IN GROUP days	WEIGHT INCREASE AFTER INJECTION 14-DAY PERIOD gm
2	100	Sham operated at first sign of xerophthalmia; injected 4 days later	400	6-10	21
5	108	Sham operated at first sign of xerophthalmia; 15 $\mu$ g 1-thyroxine daily for 4 days prior to injection	400	5-10	24
5	115	Sham operated at first sign of xerophthalmia; 150 mg thiouracil in food daily for 4 days prior to injection	400	5-11	14
6	118	Thyroidectomised at first sign of xerophthalmia; injected 4 days later	400	7-9	10

The average gains were linear to the 0.45% level of tryptophan, at which they reached a plateau. The level of 0.6% appears to be approaching toxicity and growth was depressed somewhat in this group. The linearity of gains up to the 0.45% level of supplementations would seem to imply that this point should be taken as the requirement for DL-tryptophan in the absence of nicotinic acid. Failure of the control group receiving 0.3% of DL-tryptophan and 50 mg of nicotinic acid per kilogram of diet to equal the gains of the 0.45% tryptophan group is unexplainable.

The weight gains are plotted in figure 1 d. It is obvious that more data are needed between 0.5% and 0.6% levels of supplementation in order to establish a plateau. A plateau cannot be obtained from the data of this experiment since the 0.3%, 0.35%, 0.40% and 0.45% levels of supplementation must be used to obtain a significant positive slope. A line fitted to the data of the remaining 0.5% and 0.6% levels has a significant negative slope and therefore its intersection at 0.47% with the positive slope does not necessarily designate the point at which the response is maximum. However, it does give an estimate of the requirement.

The feed required per unit gain as shown in table 7 is at a minimum at the 0.45 and 0.5% levels of DL-tryptophan. This together with the weight gains suggests that the requirement for DL-tryptophan in the absence of nicotinic acid is approximately 0.45% of the diet.

#### DISCUSSION

The data indicate that the tryptophan requirement of the baby pig on the diet used in this work is approximately 0.2% of L-tryptophan or 0.3% of DL-tryptophan in the presence of excess nicotinic acid and thus that one-third of the D-tryptophan is utilizable by the pig for growth.

The data indicate that approximately 0.45% of DL-tryptophan (equal to 0.3% of L-tryptophan) is required to supply



therefore, the work of Remington et al. ('42), and Wiese et al. ('48). They contrast, however, with that of Canadell and Valdecasas ('47), Johnson and Baumann ('47), and Kelley and Day ('48), due probably to differences associated with intestinal absorption. They contrast also with the results of Drill and Truant ('47) but an explanation for this is difficult to offer.

It is of interest to note the significantly higher ( $P < 0.05$ ) liver vitamin A storage in the group rendered hypothyroid with thiouracil (table 1), an irregularity which has also been reported in blood by Bieri ('49) and which would appear to be attributable to thiouracil *per se* rather than to the thyroid. The somewhat smaller weight increase in the hypothyroid group after injection would appear also to confirm the observations of Wiese et al. ('48) who have emphasised that when assessing the effect of drugs such as thiouracil on carotene metabolism by biological assays involving weight increases, allowance must be made for the growth-inhibiting action of the drugs themselves.

#### SUMMARY

The effect of the thyroid on the conversion of carotene to vitamin A has been studied in the rat, carotene being administered intravenously as an aqueous dispersion in Tween 40 (polyoxyethylene sorbitan monopalmitate).

Twenty-four hours after the injection of carotene into normal, hyperthyroid, hypothyroid, and thyroidectomised animals partially deficient in vitamin A, liver levels of vitamin A increased from 1.1  $\mu\text{g}$  to 11.0, 10.6, 12.8 and 10.2  $\mu\text{g}/\text{liver}$ , respectively, while blood levels of vitamin A increased from 11  $\mu\text{g}$  to 19, 17, 21 and 18  $\mu\text{g}/100\text{ ml plasma}$ , respectively.

Within 11 days of injection into completely deficient animals, ocular symptoms had disappeared from all 4 treated groups. The rate at which symptoms disappeared was approximately the same in all groups while weight increases, with the exception of the hypothyroid group which was lower, were the same also.

# SUMMARY

The relationship between the requirements of the baby pig for L-tryptophan, DL-tryptophan and nicotinic acid were studied using a hydrolyzed casein synthetic milk diet containing 28% of protein.

1. The requirement of the baby pig for DL-tryptophan is approximately 0.29% of the dry matter of the diet when an excess of nicotinic acid is present.

2. The requirement of the baby pig for L-tryptophan in the presence of an excess of nicotinic acid is 0.19% of the dry matter of the diet. The difference between the L and DL requirements implies that about  $\frac{1}{3}$  of the unnatural isomer can be used for growth.

3. The nicotinic acid requirement for maximum growth is near 20 mg per kilogram of diet when 0.3% of DL-tryptophan is present in the diet.

4. The DL-tryptophan requirement is increased to approximately 0.45% of the diet when nicotinic acid is absent.

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# EFFECT OF GENISTIN ON GROWTH AND DEVELOPMENT OF THE MALE MOUSE<sup>1,2</sup>

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The discovery of the estrogenic activity of genistein, 4',5,7 trihydroxyisoflavone (Bradbury and White, '51) isolated from subterranean clover focused attention on the distribution and physiological effects of estrogenic-like compounds in feeds. Carter et al. ('53) and Cheng et al. ('53) found that the estrogenic activity of soybean oil meal, as measured by the uterine weight of immature female mice, is due to the presence of genistin, the glucoside of genistein. Work in our laboratory (Carter et al., '55) also has shown that when genistin, isolated from soybean oil meal, was fed to mice at a level of 0.2% of the diet fewer litters were born.

The present study is concerned with the further exploration of some of the effects produced by genistin isolated from soybean oil meal. The first objective was to determine the effect of genistin on growth of the male mouse and on the testicular development as measured by testes weights and spermatogenesis. A second objective was to compare the

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## MATERIALS AND METHODS

Two separate experiments were undertaken to investigate the effects of insulin administration on vitamin B<sub>6</sub>-deficient rats. In each experiment, groups of 10 rats were employed, except that the initial groups in experiment II comprised only 8 rats each. Wistar strain rats<sup>1</sup> were used throughout. Average initial body weights were: experiment I, males 113 gm, females 110 gm; experiment II, males 134 gm, females 121 gm. Rats were housed in individual screen-bottom cages in a room maintained at  $75 \pm 2^\circ\text{F}$ . The basal diet contained 20% of casein, 20% of corn oil, and was similar to that described previously (Beaton et al., '53), except that agar was replaced with a non-nutritive cellulose.<sup>2</sup> For one week prior to the experiments, all animals received the basal diet plus pyridoxine hydrochloride at a level of 5 mg per kilogram of diet. It was estimated that each animal had about 50  $\mu\text{g}$  of pyridoxine hydrochloride per day. The same concentration of this vitamin was present in the diet fed to control groups throughout the experimental period.

In both experiments separate groups of male and female rats were used so that differences due to sex could be determined. In experiment I, the treatments were: initial controls, fasted for 18 hours and killed to determine initial composition; deprived; deprived treated with insulin; control; control treated with insulin. Insulin<sup>3</sup> administration of 4 units per day subcutaneously was begun on the 10th experimental day. All of the rats in experiment I were fed ad libitum. In experiment II, the treatments were similar except that a pair of control groups were pair fed with the untreated deprived groups; insulin was not given to either pair-fed or ad libitum-fed controls. Deprived animals given insulin<sup>3</sup> received the following dosage subcutaneously per rat per day: during the first 20 days, 4 units; 20 to 27 days, 6 units; 27 to 32 days, 4 units.

<sup>1</sup> Carworth Farms.

<sup>2</sup> Alphacel.

<sup>3</sup> Protamine zinc insulin, Toronto, 40 units per milliliter.

groups of 10 animals each and then each animal within a group was assigned at random to one of the 10 treatments until a complete replication had been formed. This process was repeated for each of the remaining weight groups.

The mice were housed individually in wire cages with screen floors.

The composition of the basal diet is given in table 1. Vitamins and methionine<sup>4</sup> were provided as reported by Carter et al. ('55). Each animal was fed its assigned daily dose of genistin<sup>5</sup> or stilbestrol premixed in 1 gm of the basal diet. After this was consumed, untreated basal diet was fed ad libitum. This procedure was repeated daily throughout the experimental period of 6 weeks.

Body weights were recorded weekly. At the termination of the experiment the mice were sacrificed; fresh weights were determined and histological studies were made on the testes, adrenals, spleen and kidneys.

#### RESULTS

Ten mice died during the experiment; 4 were receiving the 4th level (highest) of genistin, two were receiving the third level of genistin and 4 were scattered, singly, among some of the other levels of the two test substances (table 2).

A regression analysis of the weight gains as the dependent variable and the logarithm of the dosage level as the independent variable indicated that there was a significant ( $P \leq 0.01$ ) linear decrease in growth rate associated with increasing levels of genistin in the diet. In fact, on the average, the mice receiving the 4th level of genistin lost weight. On the other hand all mice receiving stilbestrol gained weight; only the group receiving the highest level of stilbestrol gained significantly less ( $P \leq 0.01$ ) than the control group (table 2).

<sup>4</sup> Vitamins and methionine were contributed by Merck and Company.

<sup>5</sup> Genistin was isolated from commercial soybean meal as described by Carter et al. ('53). The soybean meal was contributed by members of the North Carolina Feed Manufacturers' Association.

rats. Because of the increasing sensitivity to insulin, the experiments were terminated before an acute deficiency had been attained. Incipient acrodynia was in evidence; insulin administration had no obvious effect on either the time of onset or degree of the acrodynia.

TABLE 1  
*Body composition in vitamin B<sub>6</sub>-deprived and control rats*

GROUP	AVERAGE FOOD INTAKE	AVERAGE WEIGHT GAIN	COMPOSITION OF THE WEIGHT GAIN		
			Water	Fat	Protein
	<i>gm/rat/day</i>	<i>gm/rat</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
<i>Experiment I</i>					
A. Males					
— B <sub>6</sub>	13	61	37	6	15
— B <sub>6</sub> + insulin	14	97	45	28	16
+ B <sub>6</sub>	16	143	76	34	27
+ B <sub>6</sub> + insulin	16	144	74	35	28
B. Females					
— B <sub>6</sub>	11	32	14	7	11
— B <sub>6</sub> + insulin	13	58	18	26	13
+ B <sub>6</sub>	12	67	31	20	12
+ B <sub>6</sub> + insulin	15	96	34	42	15
<i>Experiment II</i>					
A. Males					
— B <sub>6</sub>	12	90	58	9	21
— B <sub>6</sub> + insulin	14	124	61	36	23
+ B <sub>6</sub> pair fed	12	145	79	35	27
+ B <sub>6</sub> fed ad libitum	15	149	87	31	30
B. Females					
— B <sub>6</sub>	10	45	21	11	8
— B <sub>6</sub> + insulin	12	74	23	35	10
+ B <sub>6</sub> pair fed	10	81	37	26	13
+ B <sub>6</sub> fed ad libitum	13	86	42	25	15

Data regarding food intakes, body weight gains, and changes in carcass composition are shown in table 1. Since only group averages are available, it is impossible to calculate the significance of differences. Cautious interpretation is in order. The purpose of insulin administration was to

As is shown in table 2, there was an increase in adrenal weights associated with the first two levels of genistin, followed by a drop in adrenal weights in animals receiving the two highest levels of genistin. All the groups of mice receiving stilbestrol showed an increase in adrenal weight as compared to the control group. There was only a slight correlation between body weight and adrenal weight ( $r = +0.027$ ).

The differences observed in kidney weights could be explained by differences in body weight. The correlation between body weight and kidney weight was  $r = +0.769$ . Necrotic areas were observed on the kidneys of animals receiving the three higher levels of genistin.

The spleen weights, table 2, were quite variable. There were no significant differences in weight of the organ that could be attributed to differences in treatment of the animal.

#### DISCUSSION

The evidence presented indicates that genistin at certain dose levels has a detrimental effect on survival, growth rate and spermatogenesis in mice. Undoubtedly the effects observed could be partially explained by the probable coincident decrease in food intake but it is unlikely that taste *per se* was a significant factor, because of the feeding procedure used. It appears, therefore, that the results obtained were due, in part at least, to an effect of genistin other than nutrient intake.

One of the questions remaining is whether or not the effects of genistin are associated with its estrogenic properties. The depressing effects of exogenous estrogens on growth and testicular development are considered to be mediated via the pituitary. All the naturally-occurring estrogens and stilbestrol apparently decrease the output of gonadotrophin and growth hormone of the pituitary (Richards and Kueter, '41; and Emmens and Parks, '47).

A comparison of the results obtained on growth and testicular development indicate that the physiological action of genistin is different from that of stilbestrol. As is shown in



tion. No such interaction was seen with respect to the alanine-glutamic transaminase. There was no general effect of insulin on the activities of either of these enzymes.

Although an analysis of variance was not possible in the second experiment due to its design, the "t-test" was used to ascertain the significance of the differences between means. In general, confirmation of the results of the first experiment

TABLE 2  
*Alterations in liver enzyme activities in vitamin B<sub>6</sub> deficiency*  
(mean  $\pm$  standard error of the mean)

GROUP	NUMBER OF RATS	CATALASE <sup>1</sup>	ALANINE-GLUTAMIC TRANSAMINASE <sup>2</sup>	ASPARTIC-GLUTAMIC TRANSAMINASE <sup>3</sup>
<i>Experiment I</i>				
— B <sub>6</sub>	20		41 $\pm$ 1.9	110 $\pm$ 3.0
— B <sub>6</sub> + insulin	14		38 $\pm$ 1.9	92 $\pm$ 3.0
+ B <sub>6</sub>	20		60 $\pm$ 2.8	115 $\pm$ 1.7
+ B <sub>6</sub> + insulin	20		59 $\pm$ 3.8	120 $\pm$ 3.2
<i>Experiment II</i>				
— B <sub>6</sub>	16	0.70 $\pm$ 0.026	37 $\pm$ 1.4	104 $\pm$ 2.6
— B <sub>6</sub> + insulin	12	0.64 $\pm$ 0.030	37 $\pm$ 1.6	99 $\pm$ 6.1
+ B <sub>6</sub> pair fed	16	0.61 $\pm$ 0.033	51 $\pm$ 2.5	106 $\pm$ 2.7
+ B <sub>6</sub> fed ad lib.	16	0.63 $\pm$ 0.025	50 $\pm$ 2.8	116 $\pm$ 2.7

<sup>1</sup> Activities expressed as milliequivalents of sodium perborate destroyed per milligram of wet tissue per hour. Method of Feinstein ('49).

<sup>2</sup> Activities expressed as microliters of pyruvate-CO<sub>2</sub> formed per milligram of wet tissue per hour. Method of Tonhazy et al. ('50) as modified by Caldwell and McHenry ('53).

<sup>3</sup> Activities expressed as microliters of pyruvate-CO<sub>2</sub> formed per milligram of wet tissue per hour. Method of Tonhazy et al. ('50).

was obtained. Deprivation of vitamin B<sub>6</sub> led to a significant depression of alanine-glutamic transaminase activity as compared to pair-fed or ad libitum-fed controls ( $t=4.83$  and  $4.10$ , both significant at the 1% level); insulin administration to deprived rats had no effect on the activity of this enzyme nor did the restriction of the food intake of control rats have any effect. The deprived rats exhibited a lower aspartic-glutamic transaminase activity than did the ad libitum-fed

## SUMMARY

When genistin was fed at levels of 9, 18, 36 and 72 mg per day per mouse, the following results were obtained:

1. An inverse linear relationship was found between the logarithm of dose levels of genistin and growth rate.

2. Genistin appeared to have a depressing effect on testes weight beyond that attributable to differences in body weight.

3. No spermatozoa were present in testes of the mice given the two higher levels of genistin.

The effects of genistin on growth and testicular development differed, both qualitatively and quantitatively from those of stilbestrol.

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FOOD INTAKE AND UTILIZATION OF LYSINE-  
DEFICIENT PROTEIN BY THE CHICK IN  
RELATION TO THE DIGESTIBLE  
ENERGY CONCENTRATION  
OF THE DIET<sup>1</sup>

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THREE FIGURES

(Received for publication December 15, 1955)

An inverse relation between food intake and the digestible energy concentration of the food is quite well established with diets adequate in all know essentials except energy (Adolph, '47; Dansky, '52; Strominger, Brobeck and Cort, '53; Hill and Dansky, '54; Peterson, Grau and Peek, '54). It is also generally accepted that the regulation of food intake is in some way related to energy needs although the pathways by which a need for energy is translated into an impulse to eat have not been established. However, the relation of energy needs to the ad libitum intake of diets deficient in factors other than energy is less clear (Carpenter, '53). With diets moderately deficient in amino acids, the poor growth and food consumption have been variously attributed to (1) a refusal to eat (an instinctive ability to recognize the deficiency), (2) a feeling of ill-being provoked by the deficiency,

<sup>1</sup> The data in this paper are taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, University of California, 1954.

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## BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1957 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this honor.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award

*Chairman, Nominating Committee:*

C. G. MACKENZIE

*Department of Biochemistry*

*University of Colorado School of Medicine  
4200 E. 9th Avenue, Denver 7, Colorado*

ferent levels of crystalline L-lysine hydrochloride. Grau ('48) found that 0.68% of L-lysine permitted the maximum growth of chicks fed a diet containing 15% of sesame seed oil meal protein. In the present experiments, sesame seed oil meal at a level of 15% of crude protein was estimated to provide 0.42% of L-lysine (Block and Bolling, '51). Thus, lysine limited growth until the total level reached 0.68%.

## METHODS

Single-Comb White Leghorn chicks of both sexes were maintained on a stock laboratory diet in thermostatically controlled, electrically heated battery brooders until two weeks of age. At that time they were uniformly distributed by weight into

TABLE 1  
*Composition of the basal 15% sesame-protein diet*

INGREDIENT		INGREDIENT	
	%		%
Sesame seed oil meal <sup>1</sup>	32.95	Folic acid <sup>2</sup>	0.0002
(45.51% or 46.0%	or	Sucrose <sup>4</sup>	1.00
crude protein)	32.61	Calcium carbonate	1.75
Crude soybean oil	2.00	Tricalcium phosphate	1.8
Choline chloride	0.20	Monosodium phosphate	1.3
Fortified fish oil	0.25	Potassium chloride	0.6
(2250 A — 300 D/gm)		Sodium chloride	0.48
Natural mixed tocopherols <sup>3</sup>	0.05	Magnesium sulfate	0.24
2-methyl-1,4-naphthohydro-		Sodium silicate	0.11
quinone diacetate	0.001	Manganese sulfate	0.015
Thiamine hydrochloride	0.0005	Aluminum sulfate	0.003
Riboflavin	0.0005	Ferrie citrate	0.003
Pyridoxine hydrochloride	0.0005	Cupric sulfate	0.0013
Nicotinic acid	0.0015	Zinc sulfate	0.0013
Calcium (d) pantothenate	0.0015	Cobalt acetate	0.00006
Biotin	0.00001	Vitamin B <sub>12</sub> <sup>5</sup>	5 µg/kg
		Glucose <sup>6</sup>	to 100%

<sup>1</sup> Kindly provided by the Pacific Vegetable Oil Corporation, San Francisco, California.

<sup>2</sup> Kindly provided by the Lederle Laboratories, Inc., Pearl River, New York.

<sup>3</sup> Kindly provided by Merck and Co., Rahway, New Jersey.

<sup>4</sup> Sucrose was used as a carrier for water-soluble vitamins.

<sup>5</sup> Type IV-34 (340 mg/gm), Distillation Products, Inc., Rochester, New York.

<sup>6</sup> Cerelease.

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lysine, the introduction of cellu flour caused little improvement in growth although in experiment 1, growth at 0.72% of lysine was significantly greater in the presence of 12% of cellu flour.

*Food, protein and lysine intakes.* The addition of cellu flour to the diets always resulted in an increased food intake at all levels of lysine (fig. 2), and the differences in food intake in response to the variations in energy level were

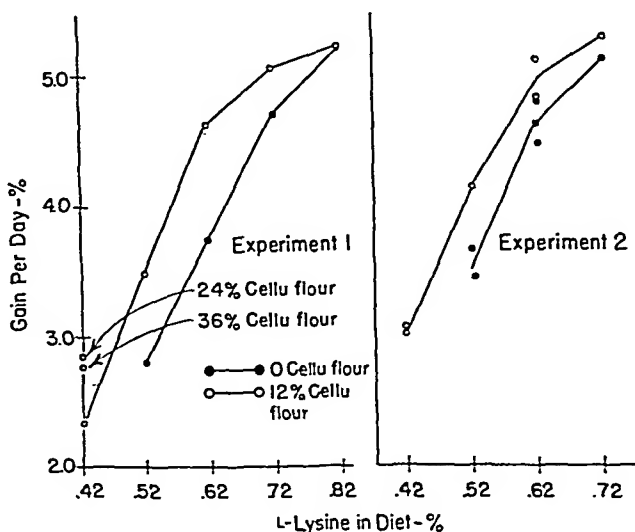


Fig. 1 Growth in relation to dietary levels of lysine and cellu flour.

apparent at the end of the second day. One-day food intakes were not measured.

In the presence of 12% of cellu flour, the food intake at a given level of lysine equalled or exceeded the intake at the next higher level of lysine in the absence of cellu flour. As food intake increased, the intake of protein and lysine likewise increased, and at any given level of lysine, the greater lysine intake occurred with the diet containing cellu flour (fig. 3).

*Energy intake.* The average estimated metabolizable energy intakes for the 18-day period are given in table 2. Until the level of lysine reached 0.82% in experiment 1 and 0.72%



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At 0.42% of lysine, an increase in the level of cellu flour from 12 to 24% resulted in a greater intake of metabolizable energy, but the energy intake decreased when the level of cellu flour was raised from 24 to 36%.

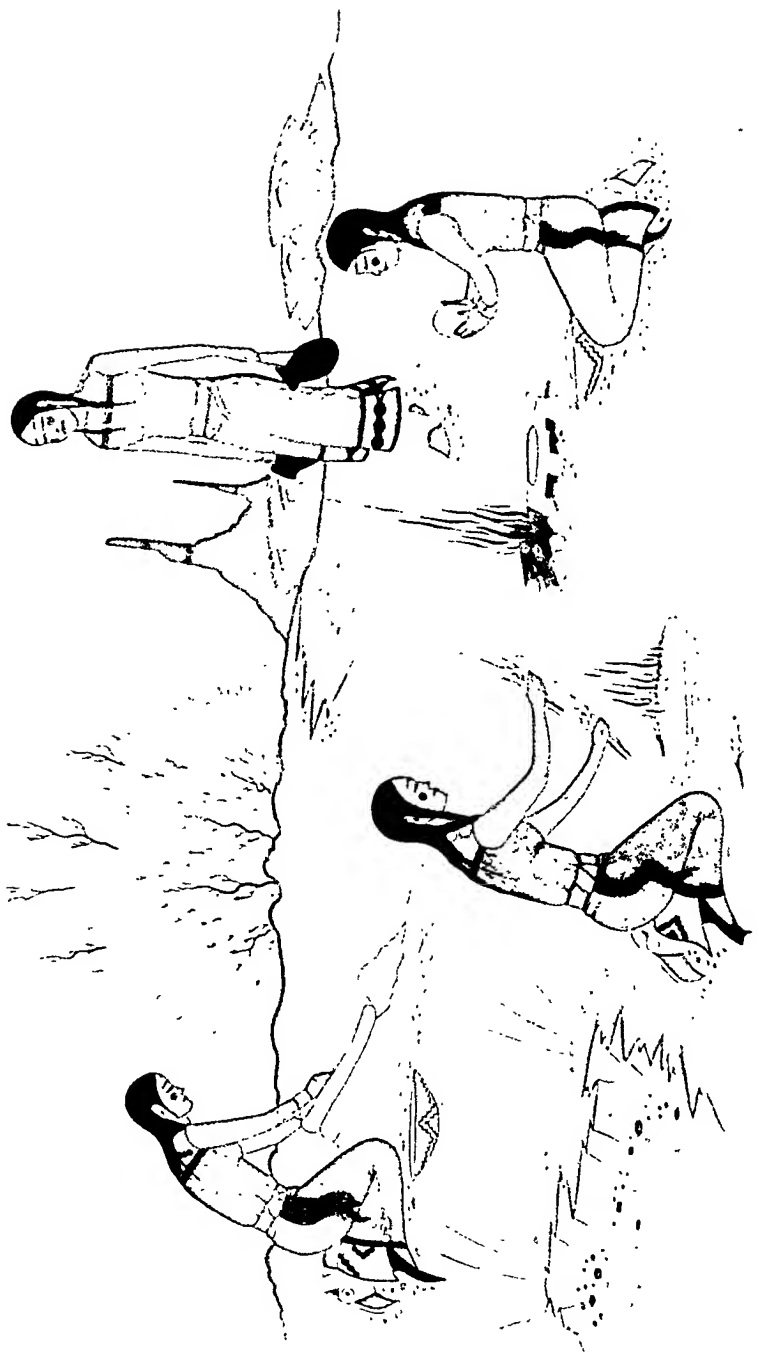
TABLE 2

*Feed efficiency and the efficiency of energy utilization in relation to the dietary levels of lysine and cellu flour*

LYSINE	CELLU FLOUR	AVERAGE FINAL BODY WEIGHT	FEED EFFICIENCY	GM GAIN
				Kcal. CONSUMED
%	%	gm	$\frac{\text{gm gain}}{\text{gm feed}}$	
<i>Experiment 1</i>				
0.42	12	152	0.181	0.063
0.42	24	169	0.180	0.073
0.42	36	168	0.156	0.076
0.52	0	169	0.248	0.075
0.52	12	193	0.252	0.088
0.62	0	202	0.298	0.091
0.62	12	245	0.308	0.107
0.72	0	246	0.361	0.110
0.72	12	268	0.345	0.120
0.82	0	272	0.381	0.116
0.82	12	274	0.353	0.122
<i>Experiment 2</i>				
0.42	12	173, 175	0.214, 0.219	0.075, 0.073
0.52	0	186, 196	0.252, 0.271	0.077, 0.083
0.52	12	217	0.272	0.095
0.62	0	234, 250	0.318, 0.338	0.097, 0.103
0.62	12	249, 266	0.317, 0.312	0.111, 0.109
0.72	0	271	0.377	0.115
0.72	12	277	0.354	0.124

*Feed efficiency and energy utilization.* Feed efficiency (grams gain per gram of food eaten) was increased by the introduction of 12% of cellu flour at the 0.52% lysine level (table 2), but was decreased at higher lysine levels (except at 0.62% lysine in experiment 1).<sup>5</sup> At all levels of lysine, how-

<sup>5</sup> The improvement in growth caused by the introduction of 12% of cellu flour at 0.62% of lysine was unusually great in experiment 1. This great an improvement in growth did not occur in experiment 2 or in other experiments not reported here.



"NAVAJO MAIDS PREPARING FOR EAT"

*Painting by Beatrice Yazz*

Grinding corn

Burning cedar branch to make  
ashes for mixing with corn meal

Carrying water

Cooking tortillas

A comparison of the basal, the 12%, and the 24% cellu flour diets at the 0.42% level of lysine illustrates the relation of lysine intake to growth rate (figs. 1 and 3). In the absence of cellu flour, mortality was 100%. When 12% of cellu flour was present, mortality was negligible, and the increased lysine intake permitted a slow rate of growth. With 24% of cellu flour, lysine intake again increased, and growth improved even more. The increased intake of the lysine-deficient diets indicates that (1) the chicks ate primarily to meet energy needs, (2) dietary palatability was not affected by a moderate lysine deficiency or by the introduction of cellu flour, and (3) the chicks did not instinctively recognize a lysine-deficient protein.

The introduction of 36% of cellu flour resulted in the greatest intake of lysine at the 0.42% level (fig. 3), but in this case, growth appeared limited by the bulk of the diet. Since food energy was always limiting, the additional protein and lysine could not be used for protein synthesis, but were metabolized to provide energy.

At 0.52 and 0.62% of the diet, lysine still restricted growth. Grau ('48) estimated that 0.68% of lysine is required in the diet for maximum growth with a level of 15% of protein. Until this level of lysine was reached, improved growth would be expected from an increase in lysine intake produced either by an increase in the lysine level or by increased food intake. The results verified this expectation: maximum growth occurred at 0.72% of lysine in the second experiment although, in the first experiment, the presence of 12% of cellu flour at 0.72% of lysine significantly improved growth.

The gains with the 15% sesame-protein diet supplemented with 0.3 and 0.4% of L-lysine equalled the gains observed when this type of semipurified diet contained 20% of fish meal protein. The occurrence of maximum gains at a level of 15% of protein without the addition of cellu flour differs from previous results from this laboratory in which maximum gains occurred with a 16% fish meal protein diet only after the inclusion of 12 and 24% of cellu flour (Peterson et al., '54).



resulted in an equal gain with an appreciably smaller energy intake. Although the equal weight gains might represent equal energy gains, it is more likely that the equal weight gains represent smaller energy gains. This possibility was suggested by the limited studies of body composition done by Peterson et al. ('54) but their data were insufficient to allow a definite conclusion. The results of Dansky ('52) and Hill and Dansky ('54), which became available during the present study, also suggested that such equal weight gains denote unequal energy gains. These authors found that the substitution of oat hulls for corn in a natural feedstuff ration resulted in weight gains equal to or greater than the gains allowed by the basal diet, but the estimated productive energy intake and the percentage of body fat decreased as the level of oat hulls increased from 10 to 40%.

#### SUMMARY

Semipurified diets containing 15% sesame protein and various levels of lysine were fed ad libitum to two-week old chicks for a period of 18 days. The substitution of 12% or more of cellu flour (wood-pulp cellulose) for an equal weight of glucose resulted in improved growth with all of the diets deficient in lysine. The improved growth was most readily attributed to the increased feed and lysine intake in response to the decrease in the digestible energy concentration of the diets caused by the substitution of cellu flour for glucose. Feed intake varied inversely with the digestible energy concentration of the diet, and energy intake appeared related to energy needs. At adequate dietary levels of lysine, the introduction of 12% of cellu flour had little effect on growth, but at all lysine levels, the presence of 12% of cellu flour reduced the estimated metabolizable feed energy required per unit of weight gain.

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To those many others who have assisted in numerous ways we are grateful. This list would become too extensive if we enumerated each person who contributed to this study. But above all we are grateful to the 1246 participants who represent the foundation of the investigation.

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ENERGY INTAKE AND  
BODY COMPOSITION OF THE CHICK IN RELATION  
TO THE DIETARY CONCENTRATIONS OF  
DIGESTIBLE CARBOHYDRATE AND  
DIGESTIBLE FOOD ENERGY<sup>1</sup>

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TWO FIGURES

(Received for publication December 15, 1955)

Improvement in the efficiency of utilization of metabolizable food energy for weight gain by chicks was observed when moderate reductions were made in the digestible energy concentration of semipurified diets containing 15% of sesame-seed protein through the substitution of cellulose (cellu flour) for glucose (Williams and Grau, '56). In these experiments, the metabolizable energy concentration of the diets was only estimated, and cellul flour was considered completely indigestible. The equal weight gains made with an apparently smaller metabolizable energy intake might represent equal gains of energy produced by more efficient utilization of metabolizable energy. On the other hand, it is well established that equal gains of weight do not necessarily represent equal energy gains (Fraps, '43; Dansky, '52; Hill and Dansky, '54). Peterson et al. ('54) found that the percentage of body fat in chicks de-

<sup>1</sup> The data in this paper are taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, University of California, 1954.

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of chicks were shifted daily to different levels in the battery. The 6 cages used occupied the second, third and 4th levels of a 5-level battery, and the chicks on each diet occupied each level 7 times in the three-week period.

Spilled food was collected with the excreta since the calorimetric determination of metabolizable food energy would not be affected by the relatively small amount of food spilled [metabolizable energy of the food = (gross food energy eaten + gross food energy spilled) — (gross energy of the excreta + gross food energy spilled)].

Some error in food intake was introduced by the failure to measure food spillage, but this was only a small percentage of the total three-week intake. The excreta were dried for 24 hours at 70°C., weighed, and pooled. No correction was made for the loss of ammonia.

At the end of the three-week experimental period, all the chicks were killed by carbon tetrachloride inhalation. The contents of the gastrointestinal tract were removed, and the carcass and intestinal tract placed in a tared beaker. Body water was determined by drying the carcass to constant weight in a forced-draft oven at 100°C. Fat was determined from the loss in weight of the dried carcass after cold extraction with ethyl ether until the extracts were fat-free. The dried, ether-extracted carcasses were then autoclaved for 6 hours in 20% sulfuric acid, and the hydrolysates were filtered and diluted to a known volume. Carcass nitrogen was determined on aliquots of the hydrolysates by the semi-micro Kjeldahl procedure. The factor 6.25 was used to estimate carcass protein from the nitrogen.

Excreta nitrogen was determined by the semi-micro Kjeldahl method with aliquots of a digest representing 0.5 gm of excreta. The moisture content of food and excreta was obtained by drying a 2 gm sample in a vacuum oven for 48 hours at 70°C. The heats of combustion of the diets and the pooled excreta were determined by combustion in an Emerson Fuel Calorimeter.

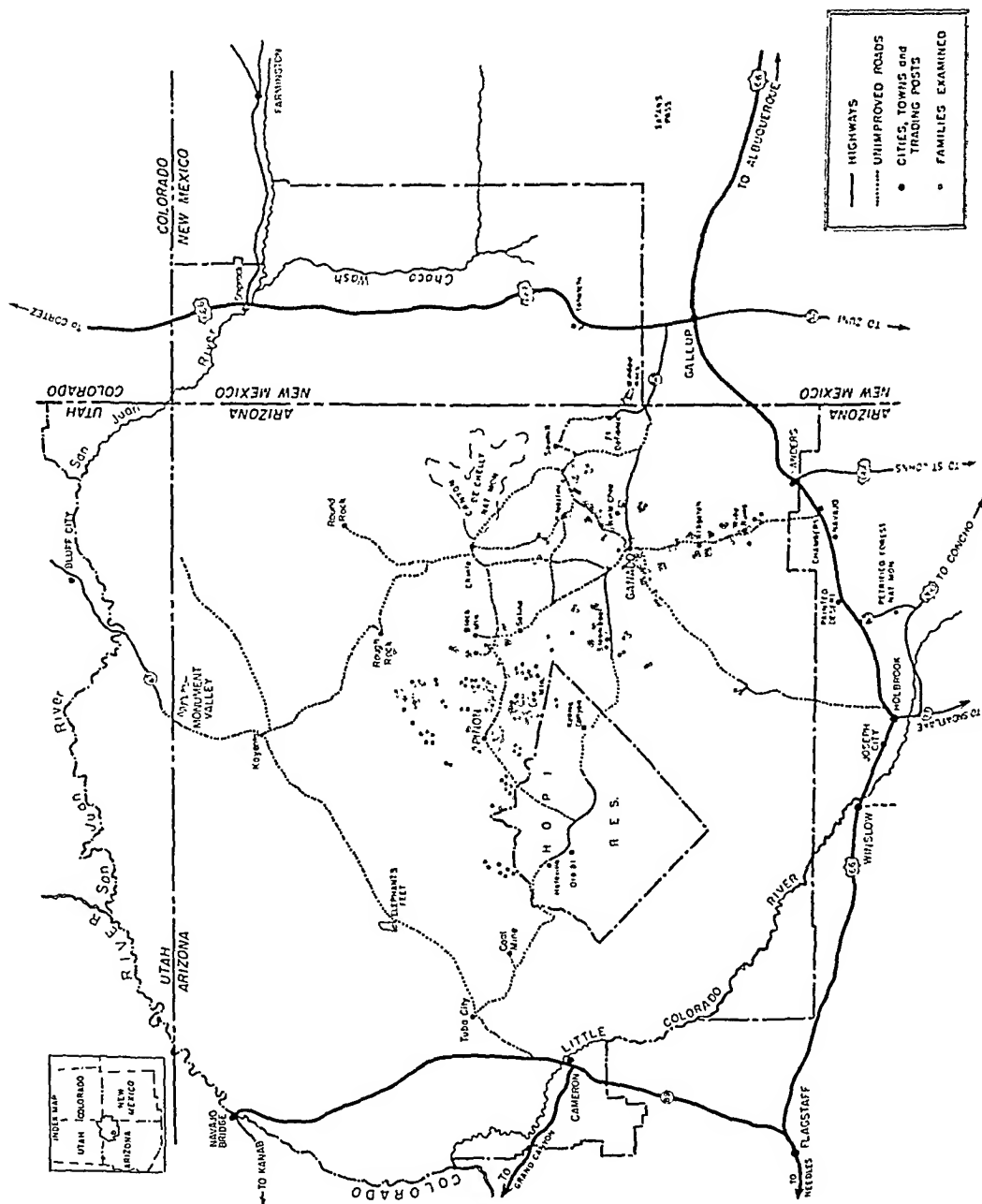


Fig. 1 The Navajo Reservation.

the introduction of cellu flour agrees with previous results with this type of diet (Williams and Grau, '56).

*Food intake.* The average cumulative food intakes are plotted in figure 2. Since the chicks were of similar size at all times, the comparison of average food intakes is valid. Up to 4 days, the intake of all three diets was nearly the same, and little adjustment in response to the differences in the

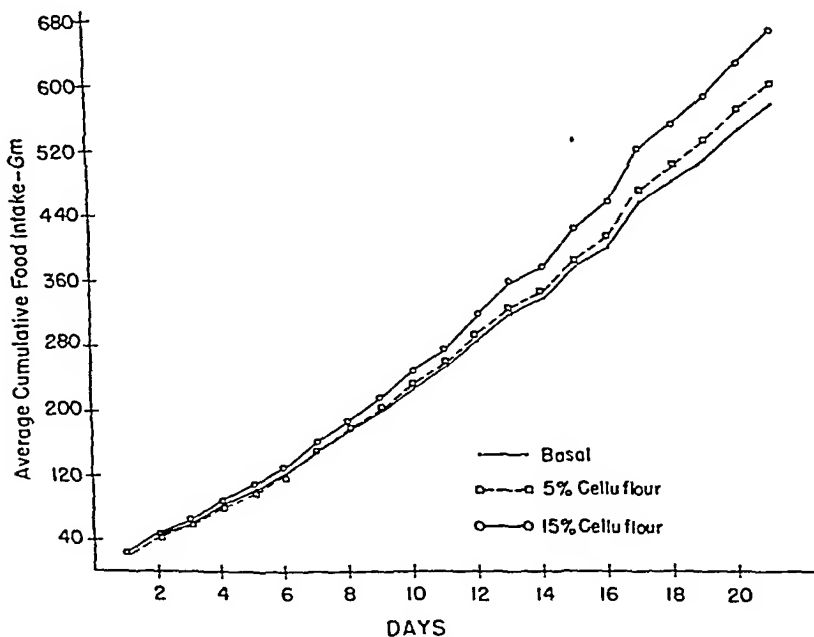


Fig. 2 Average cumulative food intakes (grams wet weight) for the 21-day experimental period.

digestible energy concentrations of the diets was apparent although the growth rates were equal. By the 5th day, the chicks fed the 15% cellu flour diet began to increase their food intake in comparison with the other two groups, and they subsequently maintained a greater intake. Little increase in the intake of the 5% cellu flour diet occurred until the 16th day. Previous experiments (Williams and Grau, '56) showed more rapid adjustments in food intake in response to greater

dian Indian by Warwick and Phillips ('54). Speculation as to environmental influences possibly contributing to these findings led to a consideration of nutrition. An initial study was begun in the summer of 1954 in the areas around Ganado and Rough Rock, Arizona. These were indecisive, but clearly indicated a deficit of basic nutritional information of "The People." The present study was designed to supply some of this basic nutritional information concerning the Navajo and was made in the area around Ganado and Pinon (fig. 1).

TABLE 1  
*Navajo agency 1950 population by age and sex<sup>1</sup>*

AGE	MALE	FEMALE	TOTAL
Under 5	5,561	5,373	10,934
5-9	5,021	5,139	10,160
10-14	4,535	4,126	8,661
15-14	12,538	13,188	25,726
45 +	4,769	4,004	8,793
TOTAL	32,444	31,830	64,274

<sup>1</sup> Source: U. S. Bureau of the Census (1953).

#### POPULATION

The population estimates from the 1950 census (U. S. Bureau of the Census, '53) are given in table 1. Certain limitations in the accuracy of these have been noted by Hadley ('55) and Kraus ('54), but they stand as the best estimates available.

The limited educational background and the high illiteracy rate are evident from the data on years of school completed by persons 25 years of age and over in 1950 (table 2). The major occupational groups of employed individuals are very nearly entirely non-professional in category and most of the employed individuals are engaged in farming and stock raising, with a smaller group serving as laborers and operatives (table 3). Data on income are probably of doubtful validity, but for 1949 almost half of the persons 14 years old and over were listed as having no income, and 76% of those reporting had an income of less than \$500 per year.

difference between the total food energy consumed and the total energy excreted per chick for the entire period.

Equal intakes of metabolizable food energy were attained with the basal and the 5% cellu flour diets, but the intake from the 15% cellu flour diet was somewhat less. The average three-week metabolizable energy intakes were 1624, 1617, and 1571 Kcal., respectively, for the basal, the 5%, and the 15% cellu flour diets. Again, the presence of cellu flour in the diet improved slightly the efficiency of utilization of metabolizable energy for weight gain (table 1). Nitrogen retention was nearly the same with all three diets and was not altered by the introduction of cellu flour. Since the nitrogen retention data indicate that equal gains of protein must have occurred, any differences in body energy gain should be reflected in the gain of body fat.

*Indigestibility of cellu flour.* The results of the present experiment confirm the assumption that cellu flour is not digested by the chick (table 1). Two different methods of approach, (1) the "digestibility" of the diet and (2) the energy content of the combined excreta, show that very little of the energy of cellu flour is available to the chick.

The "digestibility" of the diet is usually calculated from the expression  $100 (A-B)/A$  where A is the amount of a given nutrient consumed and B is the amount of that nutrient in the combined excreta. This expression, however, when applied to chicks, actually represents the "metabolizability" of the diet and indicates the amount of food available after urine and fecal losses. If cellu flour was not digested and if it did not alter the digestibility and utilization of the diet as a whole, then the difference in digestibility between the basal and the cellu flour-containing diets should equal the level of cellu flour, i.e. 5 or 15%.

On the basis of total dry matter or total organic matter, the 5% cellu flour diet contained 4 to 5% more and the 15% cellu flour diet contained 15 to 16% more non-metabolizable material than did the basal diet. The difference of 1% between

for the United States. The leading causes of death among the Navajo in 1950 were, in order: tuberculosis; enteritis, gastritis, and related diseases; "ill-defined and unknown"; diseases peculiar to infancy; influenza and pneumonia; and accidental deaths. The three leading causes of death in the general population of the United States (diseases of the heart, malignant neoplasms, and vascular lesions affecting the central nervous system) were not among the 6 leading causes of death for the Navajo.

The sociologic and anthropologic aspects of the Navajo have been reviewed on many occasions. The reader is referred to several excellent monographs (Kluckhohn and

TABLE 4  
*Closing livestock inventory 1953, Navajo reservation*

	NUMBER OF LIVESTOCK	NUMBER OF FAMILIES OWNING
Beef cattle	17,078	1,445
Dairy cattle	0	0
Sheep	387,601	6,575
Swine	0	0
Horses and mules	37,335	8,500
Poultry	0	0
Goats	68,028	5,411

Leighton, '51; Leighton and Kluckhohn, '48; Underhill, '53; Underhill, n.d.) for additional background so pertinent to one's overall appreciation of "The People."

#### FOOD PRODUCTION

Title to the Navajo Reservation is held in trust by the United States Government "for the use and benefit of the Navajo Indians." Supervision and protection of the lands are the responsibility of the U. S. Government; the lands are held in common by the members of the Navajo Tribe, are non-taxable, and cannot be sold or disposed of without consent of the Tribe and the Congress of the United States. In addition to the 15,107,983 acres of tribal lands, upwards of another 1,500,000 acres are available to the Navajo (665,021

the chicks fed the 15% cellu flour diet ( $P < 0.01$ ).<sup>4</sup> The final body water content of the chicks fed the 15% cellu flour diets was significantly greater than the body water content of the chicks fed the basal and the 5% cellu flour diets ( $P < 0.01$ ). There were no significant differences, in the final body contents of either water or fat, between the chicks fed the basal and the 5% cellu flour diets. Equivalent gains of protein were made with all three diets as the nitrogen balance data had indicated. The gains of nitrogen calculated from the results of the carcass analyses were less than the gains calculated from the nitrogen balance data. The difference may reflect the losses of nitrogen during collection of the excreta.

These results show clearly that the utilization of metabolizable food energy for body energy gain was not increased by the introduction of cellu flour at a level of 15% although the efficiency of utilization of metabolizable energy for weight gain was increased slightly.

*The regulation of food intake.* Although the food intake and digestible energy concentration of the diets are inversely related, it remains to be explained why the chicks fed the 15% cellu flour diet did not eat sufficient food to make their metabolizable energy intake equal to that of the chicks fed the basal diet since the bulk of the 15% cellu flour diet did not restrict food intake. A similar question has been asked by Hill and Dansky ('54). Also, it may be asked why the chicks fed the 5% cellu flour diet ate sufficient food to make their intake of metabolizable food energy equal to that of the chicks on the basal diet. Conversely, it might be asked why the chicks fed the basal diet did not consume *less* food.

Even if the observed differences in metabolizable energy intakes were not significant, the differences in productive energy intakes were significant.<sup>5</sup> More food energy was stored

<sup>4</sup> The significance of the differences between groups in final body composition was tested by covariance analysis of final empty body weights and final body water, fat or protein.

<sup>5</sup> Productive energy of the food = metabolizable energy of the food - heat increment of the food = energy used for maintenance.



TABLE 5  
*Production and consumption of home produced food crops on the Navajo reservation*  
 (Taken from Extension Reports of year indicated)

YEAR	USED FOR FOOD					PRODUCED				
	1941	1942	1951	1952	1953	1941	1942	1951	1952	1953
Grain (bu.)	213,183	310,401	27,344	109,212	37,746	272,865	529,525	75,066	317,909	122,456
Corn (bu.)	198,777	304,208	26,424	107,833	36,670	253,318	508,697	70,422	309,466	116,291
Wheat (bu.)	14,406	6,193	920	1,379	1,076	18,993	18,180	3,532	6,078	3,288
Oats (bu.)	..	..	....	....	....	554	2,648	1,112	2,365	2,877
Potatoes (bu.)	14,082	91,343	926	5,276	2,535	16,049	113,011	991	7,428	7,338
Beans (cwt.)	1,921	1,728	306	1,140	535	5,286	4,216	2,088	3,587	3,483
Squash (ton)	2,376	1,441	123	646	289	2,376	1,451	149	926	349
Melons (ton)		286	706	1,928	784		668	1,007	2,530	1,344
No. of families in area	8,333	9,506	12,536	12,737	13,018					

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TABLE 7  
*Annual home consumption of meat produced on the reservation*

(Data taken from Extension Reports for years indicated)

	1941	1942	1947	1948	1949	1950	1951
	Expressed as		pounds consumed				
			families producing				
Beef cattle	994,828	114,500	255,575	245,265	231,139	246,122	72,865
	700	1,217	1,304	1,320	1,347	1,385	1,420
Sheep	3,216,708	5,822,180	3,471,181	3,191,238	3,242,151	3,520,136	3,001,517
	6,210	5,324	6,862	6,756	6,819	6,972	6,848
Goats	1,274,800	1,738,765	620,890	1,148,621	1,109,310	912,636	1,004,121
	6,210	4,639	5,457	5,267	5,374	5,499	5,512
Horses and mules	403,800	490,000	91,900	634,650	537,719	289,275	457,315
	7,202	6,946	8,366	8,309	8,282	8,189	8,492

# THE EFFECT OF INTERMITTENT CONSUMPTION OF CALCIUM IN RATS<sup>1</sup>

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It appears that animal organisms are able to utilize their nutrients more efficiently when supplied at low levels in the diet. The studies of Hegsted et al. ('52) with men on low levels of calcium intake over long periods of time indicated their daily requirement for calcium was possibly considerably lower than the generally accepted high values of the conventional dietary recommendations. The study of Henry and Kon ('53) on the relationship between calcium retention and body stores in older rats suggested considerable adaptability to varying levels of intake. Recently, Hansard and Plumlee ('54) reported the physiological behavior of labeled calcium as a function of the dietary level of this element in rats. They showed that utilization efficiency decreases with increased intake of calcium and that total retention was high with low-calcium body stores.

The present paper is a report of an investigation of the effect of a schedule of intermittent feeding on calcium utilization in rats. Different groups of animals were fed the same amount of calcium; however, except for the control group this nutrient was not consumed daily, but was made available in a periodically interrupted manner. It was hoped to learn whether the mechanism of "physiological regulation" in the body (Adolph, '43) could cope with this irregular condition: reserving more calcium during the "have" period for the "have not" period.

<sup>1</sup> Journal paper no. J-2887 of the Iowa Agricultural Experiment Station, Ames. Project no. 959.

creased. Thus, in 1939, the 8,383 families consumed a minimum of 55,618 sheep and 40,859 goats (figures based on pelt purchase by trader, hence minimal) <sup>4</sup> and in 1953 the 13,018 families consumed 69,574 sheep and 41,747 goats. Herd improvement during this interval may partly equalize the actual meat consumption more nearly than is indicated by these figures based on animal units.

#### PLAN OF STUDY

This study describes the dietary habits and nutritional status of the Navajo in two contrasting regions, viz. the areas around Ganado and Pinon, Arizona, and defines the additional information necessary to obtain a valid picture which could be generalized to the remainder of the population of the Reservation.

The region around Ganado has been considerably influenced by the white culture due to the long contact (since 1904) with the Ganado Presbyterian Mission, its school and hospital, and St. Michael's Mission. Three long-established trading posts are at Ganado and the center is readily accessible to Gallup, New Mexico, and Fort Defiance, Arizona. Pinon, on the other hand, is relatively inaccessible over unimproved roads and has had no medical facility closer than Ganado or Keams Canyon. Pinon has two trading posts and a small mission. The penetration of white culture is limited. Both Ganado and Pinon are sites of recently constructed public schools.

During the months of June and July, 1955, nutrition study clinics were established at Ganado and Pinon (fig. 1). The clinic staff included physicians experienced in nutritional surveys, nutritional biochemists, nutritionists, nurses, interpreters, secretaries, drivers, and other supporting personnel. The work was initiated at Ganado on June 1. The initial period at Ganado served to orient all workers, and any member

<sup>4</sup> The 1939 Annual Extension Report shows 219,434 sheep and goats butchered, representing 4,504,580 pounds of meat for home-consumption. In addition, there was consumed 523,725 pounds of beef and 328,510 pounds of horse and burro meat.

The data of this study were analysed by the method of analysis of variance and the sum of squares for the feeding intervals were partitioned to give the mean squares for the linear, quadratic and remainder regression components (Snedecor, '46, p. 410). All expressions of statistical significance pertain to  $P = 0.05$  or less.

## RESULTS AND DISCUSSION

The initial weights of the 4 groups of animals were practically identical at 38.2 to 38.3 gm. The 6-week gains for

TABLE 1

*Effect of intermittent feeding of calcium on the composition of the femurs of rats<sup>1</sup>*

RATION GROUP	AIR-DRY WT.	ASH WT.	ASH/AIR-DRY WT.	CALCIUM	Ca/ASH	PHOSPHORUS	P/ASH
	mg	mg	%	mg	%	mg	%
1 <sup>2</sup>	571.4	250.5	43.9	88.0 (100) <sup>3</sup>	35.1	54.2 (100)	21.6
2	514.0	226.8	44.7	79.5 (90)	33.3	52.7 (97)	23.2
3	523.7	236.5	45.2	76.0 (86)	32.2	51.9 (96)	22.0
4	492.9	220.2	45.5	63.3 (72)	27.7	48.2 (89)	21.9
$s_x$ <sup>4</sup>	6.8	6.4	0.8		0.8		0.6

<sup>1</sup> Values are averages for 6 rats.

<sup>2</sup> Group 1 received 20 mg of supplementary calcium daily; group 2 received 40 mg on every second day, group 3, 80 mg every 4th day and group 4, 120 mg every 6th day.

<sup>3</sup> Figures in parentheses indicate the relative values when the value for group 1 is expressed as 100.

<sup>4</sup>  $s_x$  = standard deviation.

groups 1 to 4, respectively, were 195, 188, 199 and 192 gm with a standard deviation of 4.2 gm. The body weight gains per 100 gm of ration for the 4 groups were 30.5, 29.4, 31.1 and 30.0 gm, respectively, with a standard deviation of 0.6 gm. None of the average differences in gain was statistically significant. Hence, the feeding of equivalent total quantities of calcium but at different time intervals did not affect the body weight appreciably.

Summaries of the analyses of the left and right femurs of the animal are shown in table 1. The air-dry weight and ash

posts and discussions held with traders, as well as frequent contact with school teachers and public health nurses throughout the area. The staff of the Division of Resources of the Indian Service at Window Rock, Arizona, generously assisted through helpful discussions and by providing access to their extensive records of food production and land use on the Reservation. By combining the information so obtained a valid description of the broad dietary patterns of the population is possible.

Prior to initiation of the clinics, publicity was given to them through radio broadcasts in the Navajo language, posters at trading posts, and contact with various tribal leaders. Persons voluntarily coming to the clinic were examined and requested to return with their families. Drivers and staff made numerous personal contacts with families and transported many to the clinic. By judicious use of available local knowledge of the approximate economic position of families and of their geographic location (employing detailed district maps available for school purposes), an effort was made to obtain a representative sample of the population. The sample is biased, however, by the uncontrollable effects of refusal or willingness to participate, by the attendance of those who recognized a need for medical care (of especial influence at Ganado), and by other less identifiable factors. Nevertheless, it is our opinion that the sample provides a useful, if not absolutely representative sample of the population, and that it would be difficult under practical conditions to obtain a more representative group. The composition of the sample and a comparison of it with the 1950 census data are given in tables 8 and 9. The emphasis on adults, and especially those over 45 years of age, was a result of the interest in studying the "cancer age."

The geographical location of the families participating in this survey is shown on the accompanying map (fig. 1). Large areas of the Reservation are not included in the sampling, hence the generalization permitted is limited and additional sampling is needed.

appreciably. The femurs of the rats which received a daily allowance of supplementary calcium had the greatest air-dry weight, ash weight, and total calcium and phosphorus content. The average percentages of calcium in the femur ash were 35, 33, 32 and 28% for the 0-, 2-, 4- and 6-day intervals of calcium feeding, respectively. The average percentages of phosphorus in the femur ash bore no relation to the length of the interval of calcium feeding.

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cordingly, an evaluation of the nutritional status of a group of Navajos living on the reservation was made during the summer of 1955. The study was designed to supply basic nutritional information concerning the Navajo Indian in the areas around Ganado and Pinon, Arizona.

From 7500 individuals in 1868, the Navajo population has increased to in excess of 78,000 despite high rates of infant and pre-school mortality, endemic diseases and indescribable hardships. Among these people educational opportunities and background have been limited. A high rate of illiteracy continues. The earned income of the majority of "The People" has been reported as less than \$500 per annum.

Agriculture is based on a combination of sheep-goat grazing and cereal production. The home production of fruits and green vegetables is very limited. A comparison of the agricultural trends of the last two decades reflects the intensive program of resource development, a 7-year drought, and the changing economic status of the Navajo. As a result the production and consumption of their basic diet has shifted toward less home-grown grain, fruits, and vegetables. However, meat has remained reasonably stabilized and satisfactory.

Two contrasting areas of the reservation were selected for study. One around Ganado reflected considerable influence of the white man's culture and was readily accessible to urban centers, whereas the region around Pinon has had more limited penetration of the white man's culture, and is relatively isolated because of unimproved roads and distant medical facilities.

Dietary appraisal, biochemical assessment and physical examinations were performed on 1246 subjects. This sample is adequate to reflect age, sex, locale and gestational trends. However, large areas of the reservation are not included; hence, generalization is not permissible for all areas on the reservation.

# THE ASCORBIC ACID EXCRETION IN THE STOOL IN ELDERLY SUBJECTS<sup>1</sup>

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The excretion of ascorbic acid in the stool has been the subject of few investigations. Studies by Chinn and Farmer ('39) revealed an average excretion of 4.9 mg of ascorbic acid daily in young normal adults; similar values were reported by Martin ('41). Large variations in the intake of ascorbic acid were found to affect the fecal excretion only slightly.

No studies have as yet been reported on the ascorbic acid excretion in the stool of elderly subjects. The present investigation was undertaken with the purpose of providing such data through the determination of the ascorbic acid excretion in the feces on an ordinary diet and following a daily intake of 200 mg of ascorbic acid in tablet form.

## MATERIAL AND METHODS

The subjects included in the investigation were elderly men, who were inmates of the St. Louis Chronic Hospital. Out of a larger group of individuals 13 were selected, who were reliable and cooperative and who did not suffer from gastrointestinal disease. The ages of the men ranged between 65 and 90 with a mean of 76 years.

The ascorbic acid intake and its excretion in the stool were first studied during a one-week period, in which the individuals received the ordinary diet of the institution. The food rejected by each subject was collected, and determinations were

<sup>1</sup> Vitamin Studies in Middleaged and Old Individuals XIII. Funds provided by Hoffmann-La Roche, Inc., Nutley, New Jersey.

families in the survey; discussions were held with informants (Navajo personnel, teachers, mission workers, traders, merchants, agricultural and home demonstration workers, Public Health personnel, museum personnel, and so on), and observations made in some of the hogans, at ceremonials, and at various gatherings (sheep dipping, rodeos, and other exhibitions).

Formerly the Navajo subsisted chiefly on corn, wild game, mutton or goat meat (after the introduction of sheep and goats by the Spaniards), and a large variety of wild plants. Small patches of beans, squashes, and melons constituted the gardens. The usual drink was water, a continually scarce commodity and one which today must often be hauled great distances in barrels. A variety of native herbs were used to make occasional drinks.

Much of the early uncultivated food has been given up. The tedious harvesting of grass seeds is seldom practiced, wild game is now scarce and displaced by mutton, goat, horse and some beef. Squashes, melons, pumpkins, and some beans and potatoes are grown for home consumption. Corn is widely grown, but as the staple item in the diet is largely replaced by wheat flour purchased from the trading post. Herb beverages have been displaced by coffee, tea and soda pop. The extent of the break from the traditional and some of the influences responsible for the changes may be appreciated from the following observations.

Food is obtained from trading posts, from neighbors or is produced at home. Quantitatively there is no large dependence on wild plants as sources of food. That such plants are still consumed both in the fresh and preserved state, however, is evidenced by the fact that a member of the staff, Mrs. Pauline McKinley, procured examples of 20 such edible foods from one region. Very little wild game was reported as eaten, and that mentioned included only rabbits, prairie dogs, venison (a few families only) and porcupine. Pigs and fowl are most uncommon. The wild foods were identified as follows:

presented in table 1. In the table the daily ascorbic acid measurements have been added to obtain the total values for the 7-day period on the ordinary diet, and for the 7-day period during which the diet was supplemented with 200 mg of ascorbic acid daily.

TABLE 1

*The intake of ascorbic acid and its excretion in the stool of 13 elderly men*

SUB- JECT	AGE	CONTROL PERIOD (7 days)				ADMINISTRATION OF ASCORBIC ACID TABLETS (200 mg daily for 7 days)				
		ASCOR- BIC ACID INTAKE	WT. OF STOOL	ASCORBIC ACID IN STOOL		ASCOR- BIC ACID IN FOOD	TOTAL INTAKE OF ASCORBIC ACID	WT. OF STOOL	ASCORBIC ACID IN STOOL	
				Total	100 gm				Total	100 gm
	Yrs.	mg	gm	mg	mg	mg	mg	gm	mg	mg
1	65	68	881	10.2	1.2	320	1720	760	11.2	1.5
2	69	278	342	6.8	2.0	420	1820	472	19.3	4.1
3	74	225	395	10.4	2.6	382	1782	474	15.7	3.3
4	65	192	765	12.9	1.7	350	1750	590	11.0	1.9
5	86	248	423	14.0	3.3	222	1622	430	12.7	3.0
6	82	241	167	10.4	6.2	280	1680	423	17.7	4.2
7	90	243	221	6.6	3.0	309	1709	248	7.6	3.1
8	80	193	322	3.2	1.0	260	1660	131	5.9	4.5
9	75	279	265	5.5	2.1	364	1764	366	4.2	1.2
10	88	308	372	4.5	1.2	350	1750	379	7.2	1.9
11	69	295	329	11.4	3.5	350	1750	474	10.3	2.2
12	78	292	100	1.6	1.6	272	1672	212	3.3	1.5
13	66	139	881	8.8	1.0	185	1555	350	8.4	2.4
Mean	76	231	420	8.2	2.3	314	1714	408	10.3	2.7

It will be seen from the data in the table that an average of 8.2 mg of ascorbic acid was excreted weekly in the stool on a mean ascorbic acid intake of 231 mg, and that the average weekly excretion was 10.3 mg when the ascorbic acid intake, as the result of supplementation with ascorbic acid tablets, was increased to 1714 mg. The mean ascorbic acid concentration of the wet stool was 2.3 mg % on the unsupplemented diet, and 2.7 mg % on the supplemented diet.

is used to remove the fruit from the cactus plant. The prickly thistles on the fruit are removed by rolling in the grass. They are either eaten as they come from the plant or dried for later use. Only the pulp of the fruit is eaten, the seeds and outer skin being removed. It has a sweet flavor.

**SOUR BERRIES** (*Chil-chinbina*). These berries are usually picked in late June or July. Sometimes they are eaten raw soon after picking. Some are stone-ground into a paste and mixed with the calc-like white clay. The clay reduces the extremely sour taste of the berries. Occasionally this paste mixture is dried.

**WILD CURRANTS** (*k'injilahi*). This small mild flavored pulpy fruit is a deep orange-red in color and is ripe in July. It is eaten raw, sometimes dried.

**ORANGE BERRIES AND CLAY** (*hush-êc-dan and glêsh*). These berries are used in the same way as sour berries are. The clay is added to improve the flavor.

**SALT WEED** (*di k'onzi*). This plant is rarely used in modern times to season food. However, it is used now for the animals. It is found on the plains and in the valleys where the soil is very alkaline. The animals are driven to the areas where the salt weed grows about once every three months and allowed to graze.

**WILD SPINACH SEED** (*Cloh-dai-bi-nah*). The plants bearing seeds are gathered in August. The seeds are stone-ground into a fine meal. This meal is mixed with boiling water to make a mush and is eaten as a cereal. Sometimes it is also mixed with cornmeal to make mush.

**GRASS SEEDS** (*tlos-tsê*). These seeds are used the same way as the wild spinach seed is used.

**DRIED CURRANTS AND CLAY** (*Tsidzch-glesh*). This is the dried form of currants. The berries are stone-(matate) ground, mixed with clay and, sometimes, coarsely ground cornmeal and boiled until it forms a very thick paste. This paste is spread about an inch thick on a flat rock to dry in the sun. It is broken off in small pieces after it is thoroughly dry for eating.

**WILD ONIONS** (*T'loh chin*). The bulbs are small in size like that of the mariposa lily. They are gathered in June or July and are eaten raw or cooked with potatoes and in mutton stew.

**JUNIPER BERRIES** (*T'st-dz ch*). The berries are picked in August or early September. Some are eaten raw, others are toasted, stone-ground into a meal-like substance and mixed with the white clay. Some water is added to this batter. Cakes are made, dried in sun on a flat rock and used as needed.

**ACORNS** (*Tsé-cil-bi-nah*). These acorns grow in the timber areas on a tree which is commonly called the scrub oak. They are gathered in the fall soon after the first frost. They are roasted in either a bed of ashes or in a pan on top of the stove. They are stored in paper bags for use in winter. Occasionally some of them are stone-ground after roasting and mixed with cornmeal for making bread.

**PINYON NUTS** (*Necsh-êhéé*). They grow on the pinyon trees and are gathered in the fall when they mature. Some are sold to the trading post and others are kept for home consumption. A few families report buying them occasionally at the store.

## SHORT-TERM FEEDING STUDIES ON ACETIN FATS

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The chief components of edible fats in the United States are triglycerides of fatty acids having chain lengths of 4 to 20 carbons. Although the predominant fatty acids in most fats contain 16 or 18 carbon atoms, considerable amounts of shorter chain fatty acids are found in some fats. Thus, butterfat may contain as much as 35% of fatty acids less than 16 carbons in length. In recent years a new type of fat has been developed in which one or two of the long-chain fatty acids of the glyceride molecule are replaced with acetic acid (Baur, '54a). Such glycerides have been referred to as acetin fats or aceto glycerides.

The most striking effect of the introduction of the acetyl group into the glyceride molecule is the reduction in melting point. This effect permits the preparation of low melting fats and oils, or acetin fats, of a high degree of saturation and of a significantly increased resistance to oxidation. Acetin fats may replace normal triglycerides in any edible fat use. Thus, edible fat products including shortenings, margarines or spreads, salad oils and frying oils can be made from acetin fats and oils (Baur, '54b; Feuge, '55).

The physical properties of the acetin fats are determined by the number of acetate residues incorporated into the molecule and the nature of the long-chain fatty acids. Since there is no standard nomenclature in this field we have selected the term monoacetin fat to refer to that fat in which one of the long-chain fatty acids of a conventional triglyceride is re-

drinks, especially in the 12-ounce and quart size bottles, are consumed in large amounts, particularly during the summer.

Some seasonal pattern in grocery purchases is evident. There is an increased sale of rice, raisins, and soups in the winter, or milk in the spring and of fruits and soda pop in the summer.

TABLE 10

*Frequency of purchase of food items in 47 grocery orders observed at random  
Ganado area, July, 1955*

FOOD	NO. OF ORDERS INCLUDING THE FOOD
Sliced bread	20
Fresh fruit	18
Canned fruit or juice	17
Bologna, spiced ham, wieners	15
Shortening	15
Potatoes	14
Pop	14
Candy	13
Coffee	13
Sugar	12
Flour	11
Crackers	11
Cookies, donuts, jelly rolls, Boston cream pies	10
Milk	10
Mutton	10
Baking powder	8
Plain salt	8
French bread	7
Canned vegetables	7
Cabbage	6
Canned meats or sardines	6
Bacon or salt pork	5
Sweet rolls, coffee cake	5
Ice cream	4
Rice	3
Iodized salt	3
Chile peppers	3
Tea	3
Noodles, macaroni	2
Koolaid	2
Cheese	2
Oatmeal	2
Raisins, chewing gum, ketchup, potato chips, pancake mix, beef, waffle syrup, pinto beans, cocoa, onions, pork chops, soup, buns, each	1

TABLE 1  
*Composition of fats*

CATEGORY OF INTEREST	SERIES 1			SERIES 2			SERIES 3		
	I.V. 80 fat	I.V. 80 di-acetin fat	I.V. 1 fat	I.V. 1 di-acetin fat	I.V. 80 fat	I.V. 80 di-acetin fat	I.V. 80 fat	I.V. 80 mono-acetin fat	
Iodine value	79	52	1	1	76	54	76	69	
Saponification value	191	373	190	371	191	369	191	253	
Free fatty acid, %	0	0.5	0.2	0.6	0.1	0.2	0.1	0.2	
Acetic acid, %	0	26	0	27	0	26	0	9	
Trans fatty acids, %	19	1	0	...	21	...	21	...	
Saturated acids, %	17	...	100	...	22	...	22	...	
Oleic acid, %	75	...	...	...	67	...	67	...	
Linoleic acid, %	7	...	...	...	11	...	11	...	
Linolenic acid, %	1	...	...	...	0	...	0	...	

<sup>1</sup> Fatty acid composition that of the corresponding conventional fat with allowance made for acetic acid content.

TABLE 2  
*Percentage composition of diets*

CONSTITUENT	SERIES 1			SERIES 2			SERIES 3		
	I.V. 80 fat	I.V. 80 di-acetin fat	I.V. 1 fat	I.V. 1 di-acetin fat	I.V. 80 fat	I.V. 80 di-acetin fat	I.V. 80 fat	I.V. 80 mono-acetin fat	
	%	%	%	%	%	%	%	%	
Vitamin mixture <sup>1</sup>	5.9	5.3	5.9	5.3	5.0	5.0	5.0	5.0	
Soybean oil	2.0	2.0	2.0	2.0	..	..	..	..	
Casein	32.1	28.4	32.1	28.4	35.0	35.0	27.0	27.0	
Sucrose	28.7	33.1	28.7	33.1	4.0	..	47.0	47.0	
Salt mix <sup>2</sup>	3.3	3.2	3.3	3.2	3.0	3.0	3.0	3.0	
Cellulose	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	
Ethyl cellulose	..	..	..	..	..	4.0	..	..	
Fat	25.0	25.0	25.0	25.0	50.0	50.0	15.0	15.0	

<sup>1</sup> Each 5 gm contains: vitamin A, 1250 I.U.; vitamin D, 125 I.U.;  $\alpha$ -tocopherol, 10 mg; menadione, 0.3 mg; thiamine, 0.1 mg; riboflavin, 0.5 mg; niacin, 2.0 mg; calcium pantothenate, 2.0 mg; choline, 300 mg; para-aminobenzoic acid, 10 mg; biotin, 0.03 mg; folic acid, 0.025 mg; inositol, 200 mg; ascorbic acid, 10 mg; pyridoxine, 0.1 mg; sucrose to make 5 gm.

<sup>2</sup> Hubbell et al. ('37).



fruits or cereals. There is obviously less variety among the foods produced in the Pinon area.

### FOOD PRESERVATION AND STORAGE

Drying and salting continue as the most commonly used methods of food preservation. Home refrigeration is practically unavailable. Root cellars are in fairly common use, but the foods suitable to such storage are restricted to the root crops, melons, and pumpkins. Bags or strings of dried foods and dried salted meats were commonly noted hanging from the rafters of the hogan.

TABLE 11

*Home production of foods by 238 families in Ganado area and 167 families in Pinon area*

	NO. OF FAMILIES PRODUCING THE FOOD	
	Ganado	Pinon
Corn	216	129
Beans	132	42
Squash <sup>1</sup>	125	89
Pumpkin <sup>1</sup>	33	89
Watermelon	96	91
Other melons	35	18
Potatoes	28	13
Carrots	12	1
Peaches	12	1
Other vegetables or fruits	2	2

<sup>1</sup> Pumpkin and squash not always clearly distinguished by informant.

<sup>2</sup> No other vegetable or fruit raised by more than 7 families at Ganado and one family at Pinon.

### COOKING PRACTICES

Hogans usually contain wood-burning stoves with ovens. Camp-fire cooking is popular, especially in the summer. Foods may be baked over coals, in coals, on the surface of the ground, in the ground, in a range oven, or in a separate "bee-hive" type of bread oven. Roasting is carried out over coals or in an oven. Frying or boiling is done on stoves or over camp-fires. Cooking equipment usually consists of a grate

domly among groups within each experiment as to body weight and litter. Animals were housed individually in cages. Gains in body weight and food consumption were recorded on a weekly basis.

In some instances the coefficient of utilization of the dietary fat was determined by analyzing feces collected during the 5th and 6th week of the experiment for their total lipide content. The feces were dried, ground in a Wiley mill so as to pass a 60 mesh screen, saponified with alcoholic KOH, acidified, and extracted with petroleum ether. Since this procedure is likely to result in a loss of volatile fatty acids, several samples of the feces of the animals fed the acetin fats were analyzed separately for their acetic acid content. Only a trace was found. Thus, measurement of the egested long-chain fatty acids alone will give the true value for the coefficient of utilization of an acetin fat.

Where applicable, the data were analyzed statistically by an analysis of variance. Minimum significant differences were determined by the method of Tukey ('52). A confidence level of 0.05 was used in all such treatments.

#### RESULTS AND DISCUSSION

The performance of the animals fed diets containing 25% of an acetin fat prepared from either partially or completely hydrogenated vegetable oil and the corresponding controls is given in series 1 of table 3. It will be noted that the gain in body weight of the animals fed an acetin fat is the same as that of animals fed a conventional triglyceride of the same long-chain fatty acid composition. The growth of the animals fed the fats containing only saturated fatty acids is inferior to that of animals fed partially hydrogenated fats. This response is in part explained by the lower coefficients of utilization of the completely hydrogenated fats. When account is taken of the portion of the fat in the diet which is not absorbed, the performance of all 4 dietary groups is in much better agreement as shown by the values for absorbed caloric efficiency.

lunches include soft drinks, sweet rolls, Boston cream pies, cookies, bologna, candy bars, ice cream, and fruits and melons in season. At ceremonials, rodeos, sheep dippings, and similar gatherings meals are cooked camp-style and consist of mutton, roasted corn, tortillas or bakers' bread and coffee.

TABLE 12

*Illustrative basic meal patterns of the Navajo*

	THE FAMILY OF MEANS <sup>1</sup>	MODERATELY WELL-TO-DO FAMILY <sup>1</sup>	INDIGENT FAMILY
Breakfast —	Mutton usually, or fried potatoes	Fried potatoes or oatmeal or other cereal	Fried potatoes with onions
	Oatmeal with evap. milk and sugar	Indian biscuit or fried bread	Tortillas
	Tortillas	Coffee with evap. milk and sugar, or tea with sugar	Coffee with sugar
	Coffee with evap. milk and sugar		
Noon meal —	Stew with mutton, potatoes, onions and Chile	Roast mutton or stew	Boiled potatoes
	Tortillas or fried bread or Indian biscuit	Bread or tortillas	Tortillas
	Coffee with evap. milk and sugar	Coffee with evap. milk and sugar	Coffee with sugar
Evening meal —	Same as noon	Same as noon	Potatoes or tortillas
			Coffee with sugar

<sup>1</sup> Families with herds include goat or sheep milk or both in their diets when it is available.

The coefficients of utilization of the completely hydrogenated fats are of particular interest. The value obtained for the conventional triglyceride is of the order of magnitude that would be expected for this fat, since it is essentially all tristearin (Cheng et al., 49). The replacement in the triglyceride molecule of a portion of the stearic acid with acetic acid results in a marked increase in the amount of stearic acid that is absorbed. This high coefficient of utilization of stearic acid is of interest in the question as to whether the stearic acid content or the melting point of a fat determines its coefficient of utilization. The I.V. 1 fat which consists of about 92% stearic acid has a melting point of approximately 69°C., while the I.V. 1 diacetin fat has a melting point of 33°C. and a stearic acid content of about 62%. If the coefficient of utilization is a function of stearic acid content, not more than 40% of the I.V. 1 diacetin fat should have been absorbed. The high coefficient of utilization obtained with this fat indicates the melting point of a fat to be of more importance than the stearic acid content in determining its coefficient of utilization. However, a number of other factors, besides melting point and stearic acid content, probably play important roles in determining the extent to which a fat is digested and absorbed.

The results obtained when the I.V. 80 diacetin fat was fed at a 50% level are given in series 2 of table 3. The performance of the three groups of animals was essentially the same for all categories. The presence of ethyl cellulose in the diet did not change the growth and food consumption patterns of the animals as shown by the pair of groups fed the I.V. 80 fat. In this series, where the fats were fed at a level of 50%, a greater range in the coefficients of utilization was encountered and hence the rather large differences among groups are not significantly different. For the purposes of this experiment, the results obtained show that animals fed a diet containing 50% of an I.V. 80 diacetin fat grow as well as those fed the corresponding conventional triglyceride.

The frequency of reported consumption of foods was tabulated for 336 families from the Ganado area and for 167 families in the Pinon area. The interviews for the former group included 596 individuals; for the latter 543 persons. These tabulations are presented in table 13.

These consumption frequencies again demonstrate the basic character of the dietary: breads, cereals, fats, potatoes, meats, sweets and beverages. Evaporated milk is the type most often used, and its regular appearance in an appreciable percentage of the diets reflects the use of appreciable quantities of coffee.

Since bread and meat occupy such key positions in the diet, they deserve special mention. Corn is no longer the dominant cereal of the diet. White, yellow, blue or red corn may be eaten. Bread may be prepared from dried corn or from fresh or green corn. The latter is employed in making "kneel down bread." Blue corn bread, blue corn mush, and blue corn soup are usually prepared with the addition of cedar ashes to the ground corn at the time of preparation. Corn, because of its religious significance, continues to be used in the preparation of ceremonial cake.

Tortillas (see frontispiece) are thin, round cakes, 5 to 9 inches in diameter, made of wheat flour, water, shortening and small amounts of baking powder. They are either baked on an iron griddle or grilled over hot coals and are distinguished from fried bread which is cooked in hot fat in much the same way as a doughnut.

Mutton is the usual meat. This term may be applied to either sheep or goat meat. Indeed, flocks are often mixed, containing both sheep and goats. Relatively few families have cattle and the larger size and lack of facilities for refrigeration make cattle less convenient for home slaughtering than smaller animals.

When a sheep or goat is slaughtered the blood is retained for the preparation of blood sausage. The animal is skinned and the viscera removed and rapidly cleaned. After cleaning, the intestines are wrapped around strips of fat, broiled and

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advised to drink broth to produce milk, and that blue corn gruel is an especially appropriate food to include in the diet from the onset of labor until the end of lactation. This gruel is often advised during illness and is reputed to be a traditional source of strength. Indeed, the association of blue corn gruel with lactation is so strong that an inquiry as to the frequency of eating blue corn mush often evokes a smile or chuckle.

Bailey ('40) records that for several days following delivery restrictions are passed on the mother against eating meat, potatoes, beans and bread containing either salt or baking powder in the belief that this prevents recurrence of labor pains and aids in the healing of the baby's cord.

Infants are breast fed unless for some very pertinent reason it is impossible to do so successfully. Such instances seem rare. Where the mother cannot breast-feed the infant a member of the family may wet-nurse the child. The common basic artificial food for the infant is evaporated milk which is poured from the can into the bottle and to which an unmeasured amount of water is added. Nursing bottles are seldom sterilized but may be rinsed with a small amount of water just prior to mixing a new batch of formula. Boiled goat's milk is sometimes given and may be fed to the young infant from a spoon. In the absence of any milk, finely ground white corn may be mixed with water and fed. This practice appears to be uncommon, however.

Adult foods are added as supplements from 4 to 7 months of age without any particular pattern. Broth from mutton stew, potato mashed between the thumb and finger, and mush are commonly given. Coffee is frequently given and may be offered to child as early as 4 months. It is not an infrequent sight to observe an infant taking coffee from a nursing bottle. Members of the family are seen generously sharing their bottle of soft drink with the infant.

Milk of some sort, however, remains the basic item of the diet until more than a year of age. Indeed, children may be put to the breast until two or two and a half years of age.

# THIAMINE, PYRIDOXINE AND PANTOTHENIC ACID IN THE NATURAL RESISTANCE OF THE RAT TO A CORYNEBACTERIUM INFECTION<sup>1</sup>

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## ONE FIGURE

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This report deals with the natural resistance of the laboratory rat to spontaneous and inoculated infection by a corynebacterium (strain 197) not ordinarily pathogenic for this species.

Previous reports have shown that young pantothenate-deficient rats spontaneously develop the infection (Zucker and Zucker, '54). The corynebacterium isolated from lesions of such animals reproduces the disease when inoculated into other pantothenate-deficient rats, but does not do so in rats on a complete diet, either purified or made up of natural foodstuffs (Seronde, '54). Susceptibility increases steadily with time on the deficient diet over a period of 10 to 40 days (Seronde et al., '55). Susceptibility can be induced after a longer period on a partial pantothenate deficiency which allows continued growth (Zucker et al., '55).

<sup>1</sup> We gratefully acknowledge assistance from the National Vitamin Foundation, Hoffmann-LaRoche, Inc. and Red Acre Farm, Stow, Mass.





# B VITAMINS AND NATURAL RESISTANCE

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TABLE 1

*Plan of inoculation experiments*

	DEFICIENCIES AND DIET NUMBERS				
	Pyridoxine 2515	Calorie restricted 2520	Partial thiamine 2516	Calorie restricted 2520	Pantothenic acid 2517
					9H Strain 11C Strain
None 2520					11 11
43	11	18	14	6	
	4 killed		6 killed	6 killed	11 killed
43 killed	6 killed	18 killed	3 killed		1 at 31 days 3 at 32 days 2 at 33 days 2 at 37 days 1 at 40 days 2 at 41 days
	1 at 46 days	None	1 at 36 days 1 at 39 days 1 at 43 days 1 at 44 days 1 at 47 days	None	None
Spontaneous deaths (days on diet)	None				



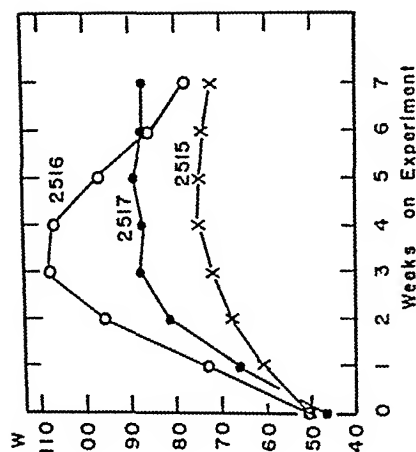


Fig. 1 Diet 2515 = pyridoxine deficiency; 2516 = partial thiamine deficiency; 2517 = pantothenate deficiency. The graph shows the average growth of rats started at three weeks of age on the three deficiencies. Each sex and strain group (6 groups in all) was averaged, and the resulting 6 means averaged to give the above curves.

TABLE 2  
Spontaneous infection with *Corynebacterium 107* in deficiencies of three B factors

DEFICIENCY	DIET	NO. OF RATS	SURVIVAL (DAYS) <sup>1</sup>		SIGNS OF DEFICIENCY (FOR GROWTH SEE FIG. 1)	CORYNEBACTERIUM INFECTION
			3 wks.	4 wks.		
Pantothenate	2517	116	57 (21-83)	77 (23-100)	Porphyrin on fur and whiskers Lousiness Adrenal hemorrhage and necrosis	Widespread
Pyridoxine	2515	17	58 (45-87)	98 (79-131)	Dermatitis of paws, lips, nose <sup>2</sup> Muscle wasting Kidney enlargement Cardine enlargement Pleural and peritoneal effusion <sup>3</sup>	Zero incidence
Partial thiamine	2516	16	54 (40-63)	56 (40-76)	Low body temperature Loss of muscular coordination Muscle wasting	Zero incidence

<sup>1</sup> All rats were allowed to die spontaneously.

<sup>2</sup> More severe and of earlier onset in the 9B strain.

<sup>3</sup> So far seen only in the 14C strain.

In the whole study, only one indisputable case of a frank vitamin deficiency disease was encountered. This was a case of mild pellagra in an elderly woman who lived alone and adhered to bizarre dietary habits. Otherwise the sample was

TABLE 15  
*Medical history data*  
Navajo study 1955

SYMPTOM	GANADO		PINON	
	No.	%	No.	%
Total entered in study	645		601	
Total with medical history	288		595	
Easy bruising or dermatitis	51	17.7	167	28.1
Burning of eyes	62	21.5	174	29.2
Photophobia	99	34.4	260	43.7
Excess lacrimation	93	32.3	247	41.5
Visual fatigue with blurring of vision	107	37.2	258	43.4
Sand, snow and night blindness	95	33.0	287	48.2
Burning, painful, tongue	12	4.2	34	5.7
Cracked lips	12	4.2	106	17.8
Angular fissures of mouth	6	2.1	91	15.3
Bleeding gums	20	6.9	163	27.4
Chronic cough	10	3.5	21	3.5
Exertional dyspnea	40	13.9	107	18.0
Palpitation	28	9.7	104	17.5
Precordial pain	37	12.8	73	12.3
Dependent edema	27	9.4	45	7.6
Anorexia	19	6.6	72	12.1
Indigestion	38	13.2	58	9.7
Chronic diarrhea, hemorrhoids with bleeding	18	6.2	71	11.9
Numbness and tingling of extremities	48	16.7	120	20.2
Syncope and dizziness	41	14.2	169	28.4
Burning feet	32	11.1	80	13.4
Leg cramps	59	20.5	149	25.0
Nervousness and irritability	65	22.6	178	30.0
A. Eye tests	633		578	
B. Either eye under 20/30 vision	190	30.0	141	24.4

free of classical deficiency diseases. This is in keeping with the recorded diagnosis of deficiency diseases found in the analysis of 60,000 admissions to hospitals on the Navajo Reservations (Salsbury, '55) and the clinical experience of

Martindale ('54), reported enlarged kidneys and hearts in pyridoxine-deficient rats. Our present data support and extend these findings and will be reported elsewhere in detail.

*Partial thiamine deficiency.* As in pyridoxine deficiency, and in contrast to the pantothenate-deficient rats, this group showed marked outward signs of physical deterioration. The animals began losing weight rapidly about three weeks after being placed on their diet. During the final week they showed muscular incoordination and lowering of body temperature. Autopsy showed marked muscle wasting. Again, no cases of corynebacterium disease were encountered, although the lungs frequently were partially atelectatic, with varying degrees of edema and occasional local hemorrhages. It is possible that these changes are attributable in part to the marked collapse of the thoracic cage and atrophy of muscles of respiration found in the thiamine-deficient rats. At any rate, these changes bore no resemblance whatever to corynebacterium infection.

In view of cardiac changes in beri-beri, and since we noted marked cardiac hypertrophy in pyridoxine deficiency, it is of interest that there was no difference in heart weight between thiamine-deficient rats and calorie controls (pair weighed).

#### RESULTS: INOCULATION EXPERIMENTS

While the observation stands, that in our experience only in pantothenate deficiency do we see spontaneous corynebacterium disease, inquiry into the mechanism of this phenomenon is obviously a fundamental and long term project. Meanwhile pertinent information has been derived from studies of artificially-produced infections where the primary barrier of the animal has been bypassed. We choose the intraperitoneal route for its convenience and apparent simplicity.

The results of the inoculation experiments are summarized in table 3. Severity of infection has been expressed on an arbitrary scale which we offer as an approximate solution to this complex problem. Values were arrived at by enumerat-

physicians at the Sage Memorial Hospital, Ganado, Arizona. Among the 60,000 records examined nutritional diseases were recorded as follows: pellagra, 10; scurvy, 2; beriberi, 2; rickets, 1; and "malnutrition," 97.

Among the so-called "subclinical signs" of nutritional deficiency disease, there were variations in the frequency with which they were recorded by individual examiners. Some group differences were associated with location, age, sex, and whether pregnant or non-pregnant. The multiplicity of these factors complicates the interpretation of the findings.

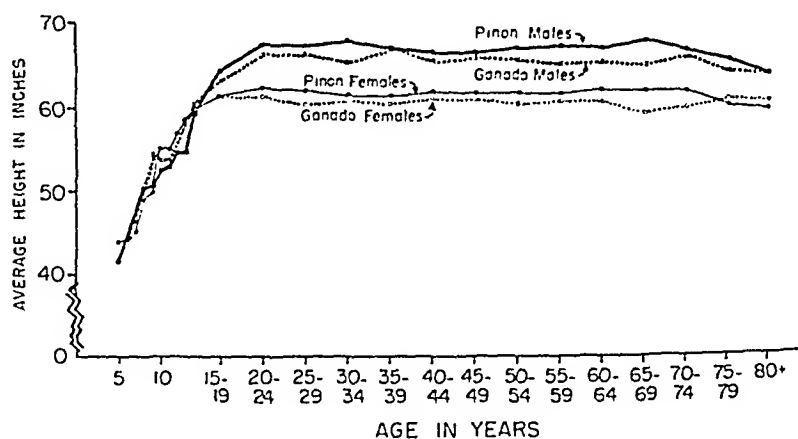


Fig. 3 Height versus age.

The correlation between these findings and laboratory data is helpful in some instances; in others, the interrelationship is indecisive.

Among the 1195 subjects (excluding the 51 pregnant women) who were examined at Ganado and Pinon, the pattern of average heights by age groups is shown in figure 3. The patterns for males and females in either location parallel each other and reveal no significant differences from 5 through 15 years. Above that age, as expected, a sex difference becomes apparent. The female reaches maximum height by the age of 17 years, and subsequently has a mean height of between 61 and 62 inches. The male reaches maximum height

TABLE 3  
*Autopsy results of inoculation experiments*

Deficiency	SEVERITY OF INFECTION † (ARBITRARY SCALE)				
	0	25	50	75	100
None 43 rats	31 6 3 1 1 . . . . .1				
Caloric 21 rats	14 3 3 2 1 1				
Pyridoxine 11 rats	1 3 1 . 1 . . . . .1	. . . . .1	. . . . .1	. . . . .1	. . . . .1
Partial thiamine 14 rats	2 1 . . 1 1 1 1 1		. . . . .1	. . . . .1	. . . . .3
Pantothenate 11 9B rats	2 . . . 1 1 . . . . .1	. . . . .2	. . . . .2		
Pantothenate 11 13C rats					. . . . .11

† Figures are numbers of rats, position of the figures moving to the right across the table represents increasing severity of infection.



TABLE 17

*Average height and weight of Navajo Indians,<sup>1</sup> by age and sex, compared to Canadian 1953 survey*

AGE (years)	NUMBER OF NAVAJO SUBJECTS		AVERAGE HEIGHT (inches)				AVERAGE WEIGHT (pounds)			
	Males	Females	Males		Females		Males		Females	
			Canada	Navajo	Canada	Navajo	Canada	Navajo	Canada	Navajo
5	4	9	41.9	41.7	41.8	43.3	40	40	41	42
6	26	29	44.6	44.6	44.2	44.3	46	45	44	43
7	15	16	47.0	47.0	46.5	45.4	50	49	49	45
8	16	36	49.1	49.5	48.9	48.9	57	54	57	55
9	5	25	51.3	51.2	51.0	50.5	63	55	62	58
10	13	13	53.5	52.8	53.3	52.9	70	62	69	66
11	16	16	55.4	53.1	55.3	54.9	77	67	77	77
12	14	21	57.4	55.7	58.2	56.9	84	74	92	84
13	10	11	59.3	56.7	60.4	58.1	94	78	102	90
14	9	14	62.2	59.7	61.3	60.4	108	89	107	102
15	10	13	64.7	61.3	62.2	60.8	119	101	112	114
16-17	14	19	66.7	65.1	62.5	60.8	136	115	120	115
18-19	9	30	68.0	64.8	62.6	61.7	144	122	124	121
20-24	27	69	67.9	67.1	62.8	61.9	151	140	124	119
25-29	29	66	68.3	66.8	62.7	61.8	160	142	126	129
30-34	28	47	68.0	66.8	62.8	60.9	167	145	130	130
35-44	37	93	67.5	66.8	62.4	61.4	167	150	135	131
45-54	45	71	66.9	66.8	61.8	61.5	164	145	144	134
55-64	55	67	66.0	66.8	61.3	61.5	161	141	147	130
Over 65	51	62	65.5	66.3	60.6	60.9	155	142	138	123

<sup>1</sup> Age was attained years; height-weight taken without shoes and with subject stripped to waist; weight recorded to nearest one-half pound. For conditions of Canadian study see Pett, L. R., and G. F. Ogilvie, *Human Biology*, 28: 177 (1956).

anisms normally operative there are found to be dependent to some extent upon adequate dietary pyridoxine and thiamine.

The complexity of the entire problem is multiplied by the probability that conditions will vary from one host species to the next. Moreover, that no two potential invading organisms need be affected in the same way is exemplified by our findings: of all the saprophytes inhabiting the rat as potential invaders (Nelson, '30), only one has so far been observed to take regular advantage of pantothenate deficiency. Our data on strain differences, illustrated in the last two lines of table 3, show that the importance of a given nutrient in natural resistance varies even within a single species. Implications of such metabolic individualism may offer an unwelcome challenge to the simpler concept of a blanket "minimum daily requirement" for every known food factor.

#### SUMMARY

A corynebacterium which produces spontaneous disease in young pantothenate-deficient rats failed to do so in similar animals deficient in either pyridoxine or thiamine, or in animals whose caloric intake was severely restricted. The disease does not appear in healthy rats on a complete diet.

Injected into animals on these various regimens, this organism caused fatal infection in all pantothenate-deficient rats of one strain, and in a relatively small percentage of pyridoxine- and thiamine-deficient rats. Another strain of rats on pantothenate deficiency proved more resistant. The complete diet, even when restricted in amount, protected the inoculated animal against serious infection.

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years of age. This relationship is such that there occurs an increase in concentration with increasing "per cent of standard weight" (table 18). This is similar to observations in pregnant women (Darby, '56).

Skin-fold measurements were performed, primarily at Pinon, on persons over the age of 15 years (except pregnant females). Three body areas were chosen: (1) skin over the posterior lateral aspect of the mid-upper arm, (2) the upper outer quadrant of the chest, and (3) either the right or left upper quadrant of the abdomen. These locations and the calipers used were in keeping with the studies of the Army

TABLE 18  
 "Per cent of standard weight" of adult male and female subjects  
 versus  
 Hemoglobin concentration

Navajo study 1955  
 (Ganado and Pinon combined — excluding 51 pregnant women)

SUBJECTS	PER CENT OF STANDARD WEIGHT			Total
	< 90.0	90-109	≥ 110.0	
	<i>Hemoglobin levels (gm/100 ml)</i>			
Males 15-44 yrs.	15.86 ± 0.15	16.09 ± 0.12	16.41 ± 0.24	16.06 ± 0.09
Males 45+ years.	15.40 ± 0.16	16.34 ± 0.16	16.75 ± 0.26	15.92 ± 0.11
Females 15-44 yrs.	14.27 ± 0.18	14.01 ± 0.13	14.24 ± 0.16	14.14 ± 0.09
Females 45+ yrs.	14.42 ± 0.14	14.84 ± 0.11	15.28 ± 0.22	14.73 ± 0.08

Medical Nutrition Laboratory (Best, '53). Despite examiner variation (table 19) in the absolute measurements, particularly at the arm site, the skin-fold thickness increased with "per cent standard weight" (table 19). Any effort to interpret further the significance of such skin-fold measurements is sharply limited by the absence of information on the influence of body configuration of various racial groups on the skin thickness measures, body composition, and their correlative significance.

Table 20 summarizes the physical findings of the sample of 1246 individuals, including the 51 pregnant and 117 lactating women.

# ANTIBODY FORMATION AND NATURAL RESISTANCE IN NUTRITIONAL DEFICIENCIES<sup>1</sup>

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Pyridoxine deficiency has been shown by Stoerk and Eisen ('46) and by Axelrod and coworkers ('47) to lead to loss of the ability to form antibodies (hemagglutinins). In a similar system Axelrod ('53) has studied the effect of other deficiencies. Agnew ('49) confirmed the pyridoxine findings with a killed culture of *B. typhosum* (*S. typhi*). Recently Pruzansky and Axelrod ('55) also studied the response to a bacterial antigen (diphtheria toxoid), and stated: "The utilization of this antigen would also relate our studies more closely to the effects of vitamin deficiencies on resistance to infection." As has been stated (Zucker and Zucker, '54), in such experiments each host species and particular kind of antigen must be considered separately. And indeed Pruzansky and Axelrod find notable differences in the effect of the same nutritional deficiencies in response to diphtheria toxoid as against the response to red cells. However, it appears that pyridoxine deficiency has the same striking effect with a variety of antigens: sheep red cells (Stoerk and Eisen, '46; Stoerk, Eisen and John, '47), human red cells (Axelrod et

<sup>1</sup>We gratefully acknowledge assistance from the National Vitamin Foundation, Hoffmann-LaRoche Inc., and Red Acre Farm, Inc., Stow, Mass.

TABLE 20  
*Physical findings among 1246 subjects*  
 Navajo study 1955

PHYSICAL FINDINGS	GANADO		PINON		TOTAL
	No.	%	No.	%	No.
Apathetic	34	5.4	14	2.3	48
Seborrhea, nasolabial	21	3.3	12	2.0	33
Seborrhea, other	2	0.3	5	0.8	7
Erythema	6	0.9	7	1.2	13
Folliculosis	0	0.0	3	0.5	3
Staring hair	1	0.2	20	3.3	21
<i>Skin</i>					
Follicular keratosis					
Grade I and II	71	11.2	67	11.1	138
Dryness and scaling	106	16.7	241	40.1	347
Crackled skin	34	5.4	81	13.5	115
Perifolliculosis	0	0.0	2	0.3	2
Acneiform eruption	12	1.9	27	4.5	39
Thick and pigmented pressure points	66	10.4	349	58.1	415
Purpura and petechia	1	0.2	7	1.2	8
Bluish-red, cold extremities	2	0.3	0	0.0	2
Pellagraform	18	2.8	35	5.8	53
<i>Eyes</i>					
Thickened conjunctive					
Grade I	179	28.2	230	38.3	409
Grade II	257	40.5	296	49.3	553
Bitot's spots	2	0.3	1	0.2	3
Circumcorneal injection	61	9.6	169	28.1	230
Conjunctival injection	199	31.3	442	73.5	641
Blepharitis	148	23.3	412	68.6	560
<i>Lips and mouth</i>					
Angular lesions and scars	62	9.8	129	21.5	191
Cheilosis	28	4.4	65	10.8	93
Pallor	0	0.0	9	1.5	9
Ulcers of mouth	0	0.0	1	0.2	1
Angular lesions and scars or cheilosis	80	12.6	167	27.8	247
<i>Tongue</i>					
Papillary atrophy	69	10.9	94	15.6	163
Papillary hypertrophy	44	6.9	35	5.8	79
Patchy denuded areas	0	0.0	0	0.0	0
Magenta colored	9	1.4	6	1.0	15

('55), and the same master cultures were used. A single batch of 48-hour broth culture, killed by the addition of 0.5% formalin and kept in the refrigerator, was used for all the vaccine and antigen preparations. On the days vaccine was required, the cells were centrifuged down, suspended in sterile saline, recentrifuged, and resuspended in sterile saline of sufficient volume to give a standard spectrophotometer reading at 700 m $\mu$  corresponding to 4.1 on the MacFarland scale (Kabat and Mayer, '48). This is about three times the optical density of the original culture.

*Procedure.* The animals were started on the various diets at three weeks of age, and caged individually in screen-bottom cages ( $\frac{1}{2}$  in. mesh). Rats on the deficient diets were fed ad libitum, while those on a restricted intake of the complete diet received a measured amount of food daily. Each of these latter was pair-weighted to a vitamin-deficient littermate so as to match its course of growth. On the 30th, 32nd and 34th day of the experiment vaccine was given intraperitoneally. For the pantothenate- and pyridoxine-deficient groups and their controls the dosage schedule was  $\frac{1}{2}$ , 1 and 1 ml; for the thiamine-deficient group and its controls it was  $\frac{1}{2}$  ml each time. After 39 days on experiment, 5 days after the last vaccine injection, blood was collected from the tail.

*Agglutination.* The serum was separated (usually without centrifuging) after standing overnight in the refrigerator. Serum dilutions were used only on the day they were made, but determinations starting out with undiluted serum were carried out over a period of up to a week after collection. Serial dilutions were made in the usual way with a twofold dilution at each step. The final solution volume was 0.4 ml (0.2 ml of serum dilution, 0.2 ml of antigen suspension) in 10  $\times$  75 mm tubes. Because of the considerable volumetric error involved in operations on this small scale, no more than 4 successive serial dilutions were employed for the final reading.

The antigen suspension to be added to the serum dilutions was prepared just like the vaccine, except that the saline

TABLE 21

*Per cent occurrence of selected physical findings by age, sex, location*  
(Pregnant and lactating women excluded)

Navajo study 1955

PHYSICAL FINDINGS	GANADO										PINON									
	Male					Female					Male					Female				
	Age	5-9	10-14	15-44	45+	5-9	10-14	15-44	45+		5-9	10-14	15-44	45+		5-9	10-14	15-44	45+	
	No.	27	31	84	76	46	36	132	130		40	35	71	78		72	40	97	73	
Dryness and scaling of skin	11	23	7	25	28	3	11	28	28		60	71	17	36		54	60	30	41	
Thick and pigmented pressure points	4	6	6	7	7	6	12	19	19		20	49	49	54		31	58	71	81	
Thickened conjunctiva	30	48	80	96	7	28	67	93	93		85	77	90	100		69	72	91	99	
Angular lesions and scars	22	29	8	1	26	11	11	3	3		48	31	13	19		43	28	8	18	
Chilosis	19	23	5	1	13	3	2	0	0		10	34	8	6		19	25	2	4	
Papillary atrophy	0	0	2	25	4	0	7	25	25		2	3	4	55		0	0	7	49	
Papillary hypertrophy	4	10	11	5	0	6	8	9	9		20	6	3	1		21	8	3	0	
Caries	41	39	38	71	59	39	54	68	68		68	69	42	92		74	30	47	90	
Edentulous	0	0	4	29	0	0	6	46	46		0	0	0	12		0	0	0	14	
Marginal gingivitis	7	52	52	57	17	14	44	41	41		60	66	66	94		68	38	60	89	
Atrophy of papillae	0	6	15	64	0	0	18	38	38		5	9	30	86		1	2	24	86	
Recession with debris	0	10	17	58	0	0	19	36	36		2	0	11	69		1	0	11	67	
Bleeding gums	4	16	6	4	7	3	6	3	3		2	6	1	5		6	0	4	0	
Blood pressure > 140/90	0	0	6	13	0	0	2	20	20		0	0	4	10		0	0	2	15	
Facial hyperpigmentation	22	19	17	30	13	8	23	38	38		80	46	55	46		50	65	43	56	
Parotid enlargement	7	0	10	13	2	3	7	12	12		5	9	25	22		4	0	9	5	

TABLE 1

*The normal agglutinin response: effect of dose*

		AGGLUTININ TITER									
		< 1	1	2	4	8	16	32	64	128	256
		Number of rats									
Vaccine per 100 gm rat <sup>1</sup>	3 × 1.0 ml									2	1
	3 × 0.45 ml										3
	3 × 0.15 ml						1		1	1	
	None	6	9	3	1						

<sup>1</sup> Stock rats aged 6 to 8 weeks, weighing 100 to 200 gm.

TABLE 2

*Agglutinin response in deficiency of pyridoxine and pantothenic acid*

		AGGLUTININ TITER									
		< 1	1	2	4	8	16	32	64	128	256
		Number of rats									
Calory deficient <sup>1</sup>								1	1	7	4
Pantothenate deficient		7	2	1		1	3				
Pyridoxine deficient		7	3								

<sup>1</sup> Pair-weighted to pantothenate-deficient rats.

Vaccine: 0.5, 1 and 1 ml. Body weight 90 to 105 gm for pantothenate-deficient rats, 60 to 75 gm for pyridoxine-deficient rats.

TABLE 3

*Agglutinin response in deficiency of thiamine*

		AGGLUTININ TITER	
		32	64
		Number of rats	
Calory deficient <sup>1</sup>		1	4
Thiamine deficient (partial) <sup>2</sup>		3	2

<sup>1</sup> Pair-weighted to thiamine-deficient rats.<sup>2</sup> 0.04 mg thiamine hydrochloride per 100 gm diet. This allows survival times comparable to these on the other two deficiencies. These thiamine-deficient rats are losing weight during the vaccination period (see Seronde et al., '56, for typical growth curve).

Vaccine: 0.5, 0.5 and 0.5 ml. Body weight 100 to 115 gm at time of first injection.



indicate extensive evidence of a B-complex deficiency state, and the levels of blood hemoglobin were adequate.

*Teeth.* Dental care and therapy are limited among the population. As a result, one could anticipate considerable gross dental pathology. There was noted an unusual attrition of the teeth, particularly in the older age group, and also a frequent occurrence of mal-position and mal-development of the permanent teeth. Examination of the teeth (tables 20, 21) revealed two extreme patterns, namely, beautiful acarious ones or rampant caries. Unfortunately, the latter condition was more common, occurring in all age groups and in over 40% of persons past 45 years. There was no detectable difference in amount of caries between Ganado and Pinon; however, adentia was more common at Ganado, and dental plates were absent at both locales. A few circumscribed areas were peculiarly low in caries; for example, a region about Low Mountain, Arizona (see map), was essentially caries-free. We did not investigate the possible role of fluoride in the water in this region. It is plausible, however, that a high fluoride water supply may have been responsible for the lowered prevalence of caries.

*Gums.* Gingival lesions were the most prevalent stigmata observed in the study (tables 20, 21, 26). Marginal redness or swelling or both were more common for all ages at Pinon than at Ganado (70.2 vs. 14.3%). With increasing age, and particularly over 45 years, secondary changes, atrophy of papillae and recessions with debris became appreciable. Bleeding gums were not frequently observed, but when present were always associated with other evidence of gingivitis. Serum ascorbic acid levels were lower in subjects from the Pinon area than in those from Ganado. This finding, together with the higher incidence of gingival lesions, might suggest that vitamin C insufficiency was responsible. However, division of all cases with gingivitis by examiner and vitamin C level in the serum failed to reveal a positive correlation. It is apparent that poor oral hygiene and inadequate dental care are major factors responsible for this high prevalence

ness of these two groups to the natural infection; nor that it had much more effect upon the course of infection artificially induced. Considering now young pantothenate deficient rats, it would appear most doubtful that the absence or reduction in agglutinin response was responsible for their unique susceptibility both to natural and artificially induced corynebacterium disease.

It may be suggested that the (partial) thiamine deficiency makes the animals subject to inoculated infection for other reasons than the deficiency per se. It is known that in extreme thiamine deficiency body temperature drops, and also that lowered body temperature may predispose to infection. However, at the time of inoculation these rats still had normal temperatures. Only towards the very end of their lives were low temperatures noted in our thiamine-deficient rats. But under these conditions of falling body temperature, the animals in the spontaneous infection experiment showed no infection.

It may also be objected that alteration of virulence of the organism may play a role equal to that of lowered resistance of the host. In particular, pyridoxine or some metabolic product for which it is responsible might be a critical requirement for the growth or invasiveness of the organism. The low susceptibility of the pyridoxine-deficient rats would then be no obstacle to the antibody hypothesis. However, we have found that rats on the pyridoxine-deficient diet for 40 days are much more susceptible to inoculation than after the standard 30 days.<sup>4</sup> Significant loss of virulence of the organisms due to the pyridoxine deficiency of the host therefore seems unlikely.

Dubos states (Dubos, '54, p. 115): "... it is plain that the microbial diseases for which there is no explanation whatever of immunity mechanisms far outnumber those where susceptibility and resistance can be explained in terms of recognized immunological reactions — cellular or humoral." The data of this report together with those of the preceding one (Ser-

<sup>4</sup> Zucker, Seroude and Zucker, unpublished data.

TABLE 22  
 "Hypertensive" blood pressure levels according to "per cent of standard weight"  
 Navajo study 1955

CRITERION	15-44 YEARS				45 YEARS +			
	Per cent of standard weight Number of subjects				Per cent of standard weight Number of subjects			
	< 90%	90-109%	110%	Total	< 90%	90-109%	110%	Total
MALE								
> 140/90	1	2	5	8	6	10	0	8
Total	52	77	25	155	79	59	13	155
Per cent hyper- tension	2	3	20	5	8	17	0	12
FEMALE								
> 140/90	0	1	5	6	17	11	8	37
Total	70	179	98	348	73	85	32	207
Per cent hyper- tension	0	1	6	2	23	13	25	18

pantothenic acid. With deficiencies of calories or thiamine, there is no significant reduction in ability to form agglutinins. No detectable agglutinins are formed in pyridoxine deficiency. In pantothenate deficiency, some animals lose their ability to form agglutinins while this capacity is impaired in others, resulting in a mean value between those for deficiencies of thiamine and pyridoxine.

Ability to make agglutinins is entirely unrelated to the degree of resistance to the live organism shown by rats on these various regimes. Therefore it is concluded that the resistance of the normal rat, which is lost in pantothenate deficiency, does not rest upon ability to form antibodies as typified by agglutinins.

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per 100 ml). Of possible significance ( $P = 0.05$ ) is the occurrence of greater variability in cholesterol level among the "hypertensives." For all age and sex groups, levels of serum cholesterol were higher in individuals whose weights exceeded 110% of the standard.

No significant relationship was found between pulse rates and body weight. The pulse rates were 6 to 7 per minute faster in women than in men, regardless of examiner. The pulse rates of the "hypertensive" group were faster than that of the "non-hypertensive" subjects (table 25).

TABLE 25  
*Pulse values, by blood pressure classification by sex and age*

SUBJECTS	BLOOD PRESSURE > 140/90	BLOOD PRESSURE ≤ 140/90	BLOOD PRESSURE UNKNOWN	TOTALS
Males, 15-44 yrs.	77.0±2.3 (8) <sup>1</sup>	71.3±0.8 (134) <sup>1</sup>	81.0±4.3 (9) <sup>1</sup>	72.2±0.8 (151) <sup>1</sup>
Males, 45 yrs. +	78.6±2.6 (18)	70.8±0.8 (132)	(0)	71.7±0.8 (150)
Females, 15-44 yrs.	85.0±6.1 (6)	79.0±0.5 (321)	94.0±6.6 (4)	79.3±0.6 (331)
Females, 45 yrs. +	81.5±2.0 (35)	76.4±0.8 (158)	(0)	77.3±0.7 (193)

<sup>1</sup> Numbers of subjects are in parentheses.

*Facial hyperpigmentation.* By the end of the initial two weeks of the study, a symmetrical bronzing pigmentation of the skin, particularly over the malar region, was noted by some of the examiners. This resembled that observed in certain oriental groups. Whether these changes are indicative of any nutritional inadequacy is undecided; certainly, exposure to the wind, dry atmosphere, and direct and reflected sunlight may determine or influence the findings. The recorded incidence of this finding varied so with observers (table 26) that any apparent age, sex, and locale variants are probably entirely artifacts. The observation that during the period of the study, adequately-fed white children playing

# SERUM VITAMIN C OF IOWA SCHOOL CHILDREN AND ITS RELATIONSHIP TO DIET AND AGE<sup>1</sup>

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ONE FIGURE

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Vitamin C has been measured in the serum of children in various geographic areas of the United States (Moyer et al., '48; Clayton et al., '53; Babcock et al., '53; Williams et al., '51; Moschette et al., '52; Storvick et al., '51; Mack and Urbach, '48; Bessey and Lowry, '47). Serum concentrations of vitamin C considerably below those which indicate tissue saturation appear to be widespread. In controlled experiments, changes in intake of vitamin C are reflected rapidly by changes in the concentration of this nutrient in fasting blood samples. Less clear-cut, but significant, relationships between intakes and serum concentrations have been observed in the surveyed populations.

Information about the nutritional status of Iowa school children with respect to vitamin C has been obtained by a survey carried out from 1949 to 1951. The present report is concerned with children from 6 to 18 years of age in Iowa. Data from the 9-, 10-, and 11-year-old children also will be analyzed along with data from children of the same age group in Ohio and Kansas and will be published separately.

<sup>1</sup> Contribution no. 16, Subproject 2, of the North Central Regional Cooperative Project NC-5, *Nutritional status and dietary needs of population groups*, in cooperation with the Human Nutrition Research Branch, Agricultural Research Service, Washington, D. C. This manuscript is published as Journal paper no. J 2616, of the Iowa Agricultural Experiment Station, Ames, Project no. 1021.

outdoors with the Indian children at Pinon also developed similar pigmentation renders doubtful the nutritional significance in these lesions. Other evidence of any meaningful insufficiency of vitamins A and B-complex was lacking among the Navajo subjects.

*Parotid enlargement.* In view of descriptions of enlargement of the parotid glands and of the association of these changes with chronic malnutrition among people in the Far and Middle East (Sandstead et al., '55), the Navajo patients were screened for parotid enlargement. The examiners obviously employed different criteria for the classification and description of the glands, and two examiners accounted for 91% of the cases at both Ganado and Pinon. This variant makes it impossible to determine age and sex relationships. In an effort to appraise the possible value of this sign as a nutritional stigma, subjects with "parotid enlargement" were compared with those for which no enlargement was recorded. These comparisons included "per cent of standard weight," concentration of total serum protein, and other physical signs of possible nutritional significance. Among the adult groups (15 years of age and older) the recording of positive findings increased with "per cent of standard weight" of the patient, and this increase was particularly apparent when the weight exceeded 110% of standard. In this study we are unable to relate enlargement of the parotid gland to evidence of nutritional inadequacies (physical signs, hemoglobin or total serum protein concentration). Further evaluation of this sign is obviously required before validating it as indicative of malnutrition.

*Pregnancy and lactation.* Of the women 19 to 44 years of age, 51 were pregnant and 117 were lactating at the time of examination. The pregnant women were distributed as follows: Ganado, 29; Pinon, 22; first trimester, 5; second trimester, 20; third trimester, 26. No subject under 19 years of age was or had been pregnant; the oldest pregnant woman examined was 44 years. This age range of reproduction commenced later than had been anticipated.

indicating a general decline in serum concentration of vitamin C with age (see below).

The mean intakes of vitamin C (table 1) remained the same or increased slightly with increasing age. Deviation of mean intakes have been reported by Eppright and others ('54b). It

TABLE 1

*Average daily intakes and serum concentrations of vitamin C of Iowa children sampled from 44 schools in 1949-1951*

AGE	NO.	MINIMUM	MEAN	MAXIMUM	STANDARD DEVIATION	MEAN INTAKE
Yrs.		mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml	mg/day
BOYS						
6	21	0.30	0.91	2.18	0.57	78
7	26	0.16	0.83	1.79	0.46	62 (25) <sup>1</sup>
8	37	0.15	0.86	2.00	0.51	74
9	35	0.15	1.07	1.85	0.57	80
10	32	0.14	0.80	1.74	0.43	74
11	24	0.33	0.88	1.87	0.48	82
12	64	0.22	0.72	1.74	0.39	87 (62)
13	27	0.15	0.70	1.81	0.50	91
14	17	0.15	0.56	1.60	0.35	89 (16)
15	14	0.19	0.50	0.88	0.22	86
16	15	0.15	0.56	1.19	0.35	115 (14)
17	7	0.21	0.60	1.94	0.32	78 (6)
18	10	0.13	0.47	1.34	0.42	91 (12)
All boys	329		0.78			82 (325)
GIRLS						
6	24	0.19	0.96	1.87	0.53	66
7	31	0.26	1.05	2.10	0.49	71
8	25	0.20	0.94	2.50	0.61	79 (24)
9	37	0.14	0.94	2.47	0.58	82
10	28	0.28	1.00	1.92	0.58	87
11	34	0.23	0.70	1.64	0.35	73 (33)
12	62	0.16	0.73	2.29	0.44	86 (58)
13	25	0.12	0.49	1.27	0.31	69
14	13	0.09	0.58	1.69	0.46	80
15	15	0.12	0.47	1.25	0.29	85
16	15	0.16	0.67	1.62	0.50	78
17	10	0.22	1.01	1.95	0.62	76
18	7	0.27	1.00	1.69	0.56	94
All girls	326		0.81			79 (320)

<sup>1</sup> Numbers in parentheses indicate difference in the number of dietary records available as compared to number of serum vitamin C determinations.



TABLE 27

*Per cent incidence of medical history data and physical findings among women 15-44 years according to reproductive status*  
Navajo study 1955

MEDICAL HISTORY DATA	GANADO				PINON				GANADO				PINON			
	Groups		NP-NL <sup>1</sup>		Lact.		NP-NL		Prog.		Lact.		NP-NL		Prog.	
	Number	62	17	16	73	73	97	22	41	30	45	27	97	22	73	73
Easy bruising and dermatitis		26	24	6	30	30	39	41								
Burning or itching eyes		26	24	31	36	36	33	36								
Photophobia		45	53	50	55	36	52	55								
Excessive lacrimation		37	47	44	50	37	48	50								
Visual fatigue with blurring of vision		44	47	31	44	44	49	64								
Sand, snow and night blindness		39	35	38	52	52	61	77								
Burning and painful tongue		5	6	0	5	5	8	27								
Bleeding gums		6	18	12	33	33	28	59								
Dependent edema		5	18	0	5	5	7	23								
Anorexia		8	12	6	18	18	23	27								
Indigestion		18	24	0	7	7	15	18								
Chronic diarrhea																
hemorrhoids with bleeding		0	6	0	16	16	11	14								
Leg cramps		13	24	19	26	26	24	68								
Either eye under 20/30 vision		28	11	26	13	13	12	41								

<sup>1</sup> NP-NL = Non-pregnant — non-lactating.

per kilogram of body weight in order to make some adjustment for differences in body size. Decreasing mean intakes per kilogram body weight and decreasing mean concentrations of vitamin C in the blood serum both accompanies increasing age (fig. 1). The vitamin C concentrations in the serum of girls older than 14 years of age were an exception to this generaliza-

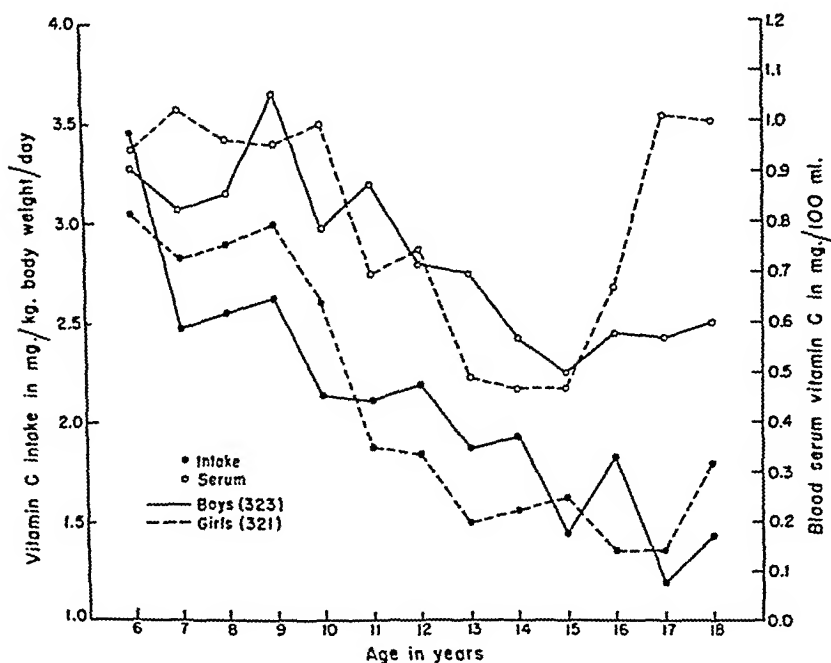


Fig. 1 Changes in mean intakes and mean serum concentrations of vitamin C with age for Iowa children sampled from 44 schools in 1949-1951 and for whom both serum values and dietary records were available.

tion and they have been handled separately in a subsequent analysis in this paper. The correlation coefficient ( $r$ ) between intake per kilogram of body weight and serum concentration of vitamin C was 0.53 for boys and 0.47 for girls. When total vitamin C intake per day and serum vitamin C were considered,  $r$  equalled 0.39 for both boys and girls. Hence, the intake per kilogram of body weight was better than intake per day for predicting serum concentration of the nutrient.

Underweight among the Navajo is relatively uncommon prior to 30 years of age. Above this age both males and females show an increasing percentage of individuals who may be considered underweight—less than either 80 or 90% of standard weight. This is in contrast to trends prevalent among adult white populations in the United States and Canada (Pett, '50). It may be worth speculation as to whether this may contribute to the observed low incidence of hypertension (blood pressure  $> 140$  mm Hg systolic/90 mm Hg diastolic) found in the Navajo subjects. The greater height among the adult men and women in the Pinon area may reflect a true hereditary trait.

The recorded changes in the skin, of dryness and scaling and hyperkeratotic lesions, are obviously influenced by the dry atmospheric conditions and by the working posture during weaving and food preparation. The satisfactory levels of vitamin A in the blood indicate that there is no widespread deficit of the nutrient which contributes to these skin changes. Both signs were more frequent among the Navajo than in the population of Newfoundland (Aykroyd et al., '49; Goldsmith et al., '50; Adamson et al., '45; Pett, '50) but less frequent than among Otomi Indians in Mexico (Anderson et al., '46a).

Continual exposure to the elements—sun, wind, direct and reflected sunlight—no doubt contributes to the high percentage of ocular lesions. The residua of trachoma are included in the findings. These ocular changes were more common in the present survey than in Newfoundland, North Carolina, or Mexico.

Gingival lesions were more frequent among this sample than has been noted in several other population groups (Aykroyd et al., '49; Goldsmith et al., '50) except for the Otomi Indians. The latter had a remarkably high intake of ascorbic acid. In the present survey the examinees at Pinon had more gingivitis and their serum level and the dietary study indicated that they had vitamin C intakes lower than the subjects examined at Ganado. Frank scurvy was lacking in both locations. Gross caries, poor oral hygiene and minimal dental

change in direction may not occur until about 17 years of age and the number of observations in this survey beyond that age was inadequate for analysis.

Total dietary intakes were grouped into (1) those diets meeting all of the Allowances recommended by the National Research Council (2) those having some nutrient less than 100% of the Allowance but none less than 67%, and (3) those

TABLE 3

*Serum and dietary ascorbic acid of Iowa survey children whose intakes of nutrients met all the National Research Council allowances or were lacking one-third of some single nutrient*

AGE	GROUP I <sup>1</sup>			GROUP II <sup>2</sup>		
	No.	Serum	Intake	No.	Serum	Intake
yrs.		mg/100 ml	mg/day		mg/100 ml	mg/day
BOYS						
6,7,8	33	0.96	90	21	0.62	46
9,10,11	25	1.07	102	25	0.68	52
12,13,14	19	0.92	137	43	0.60	68
15 +	4	0.90	158	15	0.35	61
GIRLS						
6,7,8	23	1.22	102	18	0.81	46
9,10,11	14	0.94	104	29	0.64	57
12,13,14	9	0.72	116	48	0.56	62
15 +	2	0.56	149	33	0.66	77

<sup>1</sup> Group I includes children whose nutrient intake met or exceeded the National Research Council allowances and for whom serum vitamin C analyses were available.

<sup>2</sup> Group II includes children whose nutrient intake lacked one-third of some single nutrient when compared with the National Research Council allowances and for whom serum vitamin C analyses were available.

lacking 33⅓% of the Allowance for some single nutrient. In table 3, data for groups 1 and 3 are given. Group 3 was comprised of children whose intakes of vitamin C were about one-half that of group 1; at the same time, the serum level of vitamin C was reduced to two-thirds that of group 1. Calcium was the nutrient which most frequently caused the diets to be classified into group 3 (Eppright et al. '54a), and vitamin C was the second nutrient most apt to be lacking. However,

(4) *Influence of pregnancy and lactation:* Symptoms of visual fatigue, night blindness, bleeding gums, dependent edema and leg cramps were recorded and lesions of the mouth, gums, and dependent edema were observed more commonly among pregnant subjects than among either lactating or non-pregnant women of the same age group. Hypertensive levels of blood pressure were not more frequent, and no clinically diagnosable case of toxemia was found in either center.

#### SUMMARY

1. The nutritional status of 1236 Navajo individuals has been assessed by medical history and physical examination. The study group included men, women and children, with an age spread of 5 to 92 years.

2. Frank deficiency diseases were essentially non-existent.

3. Only in the assessment of ascorbic acid nutriture was there indication of widespread insufficiency.

4. The observed minor physical changes appear to be largely a result of conditions local to the area, such as exposure to the elements in the semi-arid environment.

5. The patterns of occurrence of the various findings are described.

## SUMMARY

Data useful in defining the nutritional status with respect to vitamin C have been obtained from about 650 boys and girls in Iowa towns and cities. Decreasing concentrations of vitamin C in the serum (except girls over 14 years of age) and decreasing intakes of vitamin C per kilogram body weight were associated with increasing age. From 15 years of age, the concentration of vitamin C in the blood serum of girls increased although there was no comparable change in intake. More children over 12 years of age than children under 12 years of age were classified as poor or fair according to the categories suggested by Bessey and Lowry.

The calculated intake of vitamin C from vitamin C-rich foods only was as good as total vitamin C intake for predicting blood serum concentrations of this nutrient; intake per kilogram body weight was a somewhat better predictor of serum concentrations than was total intake per day. None of these measures of intake was a precise predictor of the vitamin C concentration in the serum. Mean intakes of vitamin C similar to the Recommended Allowances were associated with mean serum concentrations lower than 0.8 mg% in children beyond 11 or 12 years of age.

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trifugation. Surplus plasma from oxalated samples was combined with serum for estimation of cholesterol, carotene or vitamin A, but not for ascorbic acid.

The laboratory at Pinon completed the estimates for hemoglobin and of total serum protein on samples from that clinic. Serum aliquots for estimation of vitamin C were precipitated with 6% metaphosphoric acid, and, along with serum for the other estimations, refrigerated and transported to Ganado for completion of the determination. All samples obtained at Ganado were processed in the laboratory there.

The particular laboratory procedures employed were selected on the basis of convenience and dependability under field conditions and with a view to allowing for valid comparison with a number of similar studies on population groups. The methods were as follows:

*Hemoglobin and total serum protein.* The copper sulfate specific gravity method of Phillips and Van Slyke ('45) was used, the specific gravity intervals of 0.004 adopted and gravities recorded to 0.001 unit. The recommended correction was made for oxalate where present. When the sample was insufficient for determination of total serum protein the average value of 7.2 gm per 100 ml was used to calculate the hemoglobin content. Personnel limitations dictated that during the first week of the clinic at Pinon and for almost all children throughout the study at that clinic there was but simplified screening of hemoglobin level by determining whether the concentration was above or below the two limits of 12.3 or 14.2 gm/100 ml, assuming an average total serum protein of 7.2.

*Serum ascorbic acid.* A photoelectric procedure (Association of Vitamin Chemists, '51; Mindlin, '38; Bessey, '38) which employed 2, 6-dichlorophenolindophenol as an indicator, metaphosphoric acid as a protein precipitant, and citrate buffer was used. These reagents were prepared in deionized water which was obtained by passing distilled water through a mixed bed ion exchange resin. All analyses were stabilized immediately and completed within 4 days.

# FURTHER STUDIES ON THE EFFECT OF AUREOMYCIN ON THE APPARENT UTILIZATION OF VITAMIN A BY THE RAT

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ONE FIGURE

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In a previous paper it was postulated (Murray and Campbell, '55) that, while aureomycin increased the response to vitamin A as measured by the vaginal smear assay, it did not do so by increasing absorption of the vitamin from the intestinal tract. This theory was based on the assumption that all vitamin A had disappeared from the gut contents within 24 hours after administration of an oral dose. While this assumption seemed justified, more direct evidence was required. The purpose of this paper is to report (1) data on the absorption and distribution of doses of vitamin A in depleted rats and (2) further observations on the effect of aureomycin on vitamin A utilization.

## METHODS

The vaginal smear technique and diets were those previously described (Murray and Campbell, '55). Chemical assays for vitamin A were carried out according to the method of Ames, Risley and Harris ('54). For organs in which only small amounts of vitamin A were expected, a known amount of vitamin A acetate was added, and then subtracted from the total found by analysis.





of this experiment, shown in figure 1, it is apparent that aureomycin had no measurable influence on the distribution of vitamin A. It is also evident that the intestinal contents were free from vitamin A within 24 hours after an oral dose. The assumption that aureomycin could not influence the absorption of vitamin A from the intestine when it was fed only 24 hours after dosing, was therefore supported. The much smaller doses used in the vaginal smear assay might be expected to disappear from the gut contents even more quickly. As has been noted previously by Popper and Volk ('48) the gut wall held a small amount of vitamin A for periods up to 72 hours.

*Effect of aureomycin on subcutaneous doses.* The use of subcutaneous doses of vitamin A offered another approach to the study of the mode of action of aureomycin. Two vaginal smear assays were conducted in which half the rats received aureomycin (66 mg/kg) in their diets from two days before dosing until the end of the test. Each assay comprised 60 rats and was identical to those described previously (Murray and Campbell, '55) except that the doses were administered by subcutaneous injection rather than orally. In one of these assays the doses were corn oil dilutions of vitamin A acetate while in the other the doses were made by diluting a commercial aqueous dispersion<sup>1</sup> of vitamin A. Aureomycin increased the response to vitamin A in each of the assays. The increase in response amounted to 18.2% (limits at  $P = 0.05 = \pm 11.6\%$ ) when the doses were given in an aqueous dispersion, and to 16.3% (limits at  $P = 0.05 = \pm 25.0\%$ ) when oil solutions of vitamin A were used. Aureomycin therefore increased the response to subcutaneous doses of vitamin A by about the same amount as has been reported (Murray and Campbell, '55) in the case of oral doses. This supports the theory that aureomycin does not affect the response to doses of vitamin A by increasing the proportion of the dose absorbed from the intestine.

<sup>1</sup> Aquasol A, U.S. Vitamin Corporation.

groups. However, the usual increase in values was found in the 10- to 14-year-old children, and there was a significantly increased proportion of girls 10 to 14 years of age with hemoglobin concentrations above 14.2 gm per 100 ml (table 30). These results are comparable to the means and distributions of values found by Anderson et al. ('46a) among the Otomi Indians. The Navajo children had values 1.0 and 2.0 gm higher than do white or Negro children respectively in North Carolina (Milam and Anderson, '44) and than white children in Nashville (Darby et al., '47).

TABLE 30  
Percentage of hemoglobin limits among 246<sup>1</sup> Navajo children  
June-July 1955  
(Ganado and Pinon combined)

Number	MALE	FEMALE	MALE	FEMALE
	5-9 Years		10-14 Years	
	46	87	52	61
Hemoglobin gm/100 ml	%	%	%	%
Less than 12.3	7	5	6	7
12.3-14.2	59	61	50	36
Greater than 14.2	35	34	44	57

<sup>1</sup> Included are 114 children from table 29. The remaining 132 children from Pinon were screened assuming an average plasma protein of 7.2 gm/100 ml.

It is apparent that any problem of iron deficiency or, indeed, a lack of any nutrient essential for hemoglobin formation in this population is minimal. Insofar as hemoglobin levels are concerned, the group of men more nearly resemble the Olympic athletes reported on by Berry et al. ('49) than they do a deficient population. The consistently higher average values at Pinon, the area less sophisticated because of its greater isolation, than at Ganado seems logically attributable to the slightly greater altitude at which the families in this area lived.

The values for total serum protein are consistent with those of healthy groups — only 4 subjects (three adults and

any influence on the utilization of vitamin A in the presence of aureomycin. It was felt that this might furnish some further clue to the mechanism of the action of aureomycin. The following changes were made in the vitamin A-free diet: (1) starch was replaced by sucrose; (2) vitamin mixture, the composition of which is shown in table 2, was added; (3) folic acid was added at the rate of 8 mg/kg; (4) ascorbic

TABLE 2

*The effect of changes in the vitamin A-free diet and of aureomycin on the utilization of vitamin A*

DIET	NUMBER OF DAYS TO BECOME DEFICIENT AFTER AN ORAL DOSE OF VITAMIN A									
	A-free	A-free + aureo- mycin	Sucrose	Sucrose + aureo- mycin	Vita- min mix- ture <sup>1</sup>	Vita- min mix- ture <sup>1</sup> + aureo- mycin	Folic acid	Folic acid + aureo- mycin	As- corbic acid	As- corbic acid + aureo- mycin
Test										
1	18.3	19.3	19.1	19.3	17.3	18.8	19.1	19.4	18.8	18.3
2	16.7	17.5	16.1	16.6	16.9	17.4	16.6	16.8	17.0	16.6
3	15.9	16.6	15.4	15.1	16.6	17.2	17.0	17.0	16.4	15.7
4	15.6	17.0	15.3	15.3	16.4	17.5	16.5	16.8	15.7	14.5
Mean	16.6	17.6 <sup>2</sup>	16.5	16.6	16.8	17.7 <sup>2</sup>	17.3	17.5	16.9	16.5

<sup>1</sup> The composition of the vitamin mixture was as follows (amounts in milligrams per kilogram of diet): thiamine 20, riboflavin 40, niacin 80, pyridoxine 20, calcium d-pantothenate 80, inositol 800, choline 4000, folic acid 8, para amino benzoic acid 40, vitamin B<sub>12</sub> 16.

<sup>2</sup> Significant at  $P = 0.05$ .

acid was added at the rate of 2 gm/kg. Each of these diets was fed to 10 vitamin A-deficient rats, and 10 others were given the same diets plus 66 mg of aureomycin per kilogram. After two days all rats were dosed with 100 I.U. of vitamin A and the number of days that elapsed until each rat again became deficient, was recorded. Deficiency was judged by an examination of the vaginal smears. This test was repeated twice with the same groups of rats after which the rats were distributed randomly to form the groups of tests 3 and 4. The results

of the individual values was wide. Four women (two non-pregnant, non-lactating, one each pregnant and lactating) had levels below 40 I.U. per 100 ml, i.e. unquestionably low values which are usually associated with measurable changes in retinal function. Three men and 16 non-pregnant, non-lactating women had levels below the less rigid, widely used "limit of normal" of 70 I.U. per 100 ml. Despite the failure to detect clinically manifest avitaminosis A among this group, one must conclude that a small portion of them fails to ingest sufficient vitamin A or its precursors. These low values are masked in considering only means, since an appreciable number of samples contained 320 I.U. per 100 ml or above—no doubt reflecting the recent ingestion of visceral meats rich in this vitamin.

The distribution of carotene measurements was skewed toward the low side; accordingly, the values are presented as percentiles (table 28). These levels reflect an unusually low consumption of carotene-containing foods, and are similar to those observed in Norris Point, Newfoundland (Goldsmith et al., '50) where, however, the average vitamin A values were lower. Although carotene levels of the Navajo are lower than are those of the Otomi Indian (Anderson et al., '46a), the vitamin A status of the former is decidedly better. Indeed, the mean vitamin A level of the Navajo is higher than that recorded for either white or Negro southern population groups (Milam and Anderson, '44) while the carotene levels of the blood are decidedly lower.

No general decrease in serum vitamin A occurs in the older age groups, such as is reported for older white subjects (Nutrition Reviews, 14, '56). Furthermore, the depression of the vitamin level in pregnancy (Darby et al., '53b) observed in white groups was not manifested. The rise in serum carotene level during pregnancy and the subsequent fall during lactation were observed.

It is evident that the Navajo has a low carotene, predominantly (preformed) vitamin A intake and that this intake is adequate in most instances. There is, however, a small per-

to have any influence on vitamin A in the stomach, one would expect the effect to be much more marked after an oral dose than after a subcutaneous injection. This, however, was not the case. Furthermore, none of the vitamin A was found in the intestinal contents or walls.

The observation that dietary sucrose and ascorbic acid are capable of influencing the action of aureomycin has been made by others, although not with regard to vitamin A utilization. Stokstad, Jukes and Williams ('53) reported that aureomycin was more effective in promoting growth in chicks when the starch of the diet was replaced by sucrose. Daft and Schwarz ('52) found that either aureomycin or ascorbic acid prevented the appearance of deficiency symptoms in rats fed diets devoid of riboflavin or pantothenic acid. It is known (Monson et al., '54) that the intestinal synthesis of folic acid is increased in the presence of aureomycin. This may be the mechanism by which added folic acid eliminates the effect of aureomycin, but folic acid itself had no influence on the vitamin A assay. The fact that aureomycin had no influence on the vitamin A assay when sucrose, ascorbic acid or folic acid were added to the diet, suggests that intestinal bacteria are involved. Thus, aureomycin could increase the response to doses of vitamin A by eliminating bacteria which would otherwise destroy part of the dose, or by promoting the growth of bacteria which are capable of synthesizing vitamin A. Partial destruction of the dose can be ruled out because aureomycin was effective even when fed after the vitamin A dose had left the intestine. Intestinal synthesis remains a possibility. Luckey ('55) has recently presented evidence that dietary antibiotics stimulate the growth of germ-free chicks by direct action on the tissues. It is impossible to say what part, if any, such a mechanism has in the work reported here.

#### SUMMARY

Further evidence has been obtained which indicates that aureomycin does not exert its effect on the vaginal smear assay for vitamin A by increasing the absorption of the dose.

mention as another example of the relationship between lipid-soluble constituents in serum, an association which has been previously noted (Darby, Cannon and Kaser, '48; Darby et al., '49; Darby et al., '53b; Ferguson et al., '55). The similar behavior of carotene, cholesterol and tocopherols under a variety of conditions and the occasional divergence of vitamin A is probably indicative of similar transport mechanisms for these three factors so often associated, and of a rather different mechanism in the instance of vitamin A.

TABLE 33

*Serum cholesterol levels, according to levels of serum vitamin C*  
Navajo groups, June-July 1955

SERUM VITAMIN C LEVEL (mg/100 ml)	SERUM CHOLESTEROL (mg/100 ml)					
	Ages 15-44 yr.		Ages 45+ yr.		Lactating	
	No.	Av. $\pm$ S.E.	No.	Av. $\pm$ S.E.	No.	Av. $\pm$ S.E.
0.00-0.09	44	210 $\pm$ 7	97	233 $\pm$ 5	43	208 $\pm$ 7
0.10-0.19	62	218 $\pm$ 6	70	231 $\pm$ 5	27	212 $\pm$ 11
0.20-0.29	43	212 $\pm$ 7	44	242 $\pm$ 7	12	208 $\pm$ 14
0.30-59	89	212 $\pm$ 4	72	231 $\pm$ 6	9	224 $\pm$ 24
$\geq 0.60$	78	219 $\pm$ 6	29	242 $\pm$ 8	11	224 $\pm$ 22
TOTAL	316	215 $\pm$ 3	312	234 $\pm$ 3	102	212 $\pm$ 5

The ascorbic acid levels in the serum were low in the majority of cases in all groups. The distribution was skewed and, accordingly, the data are presented in percentiles (table 28). It is strikingly clear that those in the 90th percentile were abundantly nourished insofar as vitamin C is concerned.

The data permit certain generalizations; at least half of all groups, and a larger portion of several, have a vitamin C level below 0.3 mg per 100 ml. More low values occur among the older adults and among lactating or pregnant women. The values from the Pinon area are lower than are those for similar groups around Ganado — a finding consistent with the results of the physical examination of a greater incidence of gingivitis in the Pinon group. From these serum levels one may conclude that some half of the people had a dietary intake of ascorbic acid of not more than 30 mg. The low nutriture

# THE BIOLOGICAL UTILIZATION OF THE PALMITIC ACID ESTERS OF PANTOTHENIC ACID<sup>1</sup>

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## INTRODUCTION

Poor utilization of orally administered pantothenic acid (Henderson et al., '42; Silber, '45) has been shown to be due to the ready excretion of the calcium and the sodium salts into the feces (Nelson et al., '47; Rubin et al., '48), and to the possible destruction of the vitamin in the gastric juice (Rubin, '48; Rubin et al., '48). Since the long-chain fatty acid esters of pantothenic acid (Sakuragi and Kummerow, '56) are soluble in fats, the absorption and the excretion of the pantothenic acid moiety would be different from those of the water-soluble forms. Improved stability of the active fragment may also be expected because of less contact of the fat-soluble esters with the gastric juice.

In the present study, the biological utilization of the palmitic acid esters of pantothenic acid was investigated with rats. So called fat-soluble derivatives of pantothenic acid such as the acetate, the p-nitrobenzoate or the carbobenzoxy derivative have been prepared (Stiller et al., '40; Harris et al., '41; Wooley, '45). Quantitative evaluation of the biological activity of these preparations, however, was not complete (Williams et al., '50; Robinson, '51; Sebrell and Harris, '54).

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ethyl dipalmitoxypantothenate (I) and pantothenyl tripalmitate (III) respectively.

*Growth response of the pantothenate-depleted rats to a single dose of various preparations.* The male weanling rats which were used in this experiment had been kept for 4 weeks on a pantothenate-free ration (table 1, I). One per cent sulfa-

TABLE 1  
Composition of the basal ration

	DIET I	DIET II <sup>1</sup>
Glucose <sup>2</sup>	68 gm	64 gm
Vitamin-free casein	18 gm	24 gm
Corn oil	10 gm	8 gm
Wesson salts	4 gm	4 gm
Vitamins per 100 gm ration		
Choline chloride	200.0 mg	100.0 mg
Inositol	100.0 mg	40.0 mg
Niacin	10.0 mg	2.0 mg
Riboflavin	0.9 mg	1.0 mg
Thiamine hydrochloride	0.45 mg	0.5 mg
p-Aminobenzoic acid	0.3 mg	1.0 mg
Pyridoxine hydrochloride	0.15 mg	0.5 mg
Menadione		0.5 mg
Folic acid	0.08 mg	...
Biotin	0.4 µg	...
Vitamin A	160 I.U./week	
Vitamin D	1.6 µg/week	
Vitamin E	259 µg/week	

<sup>1</sup> Composition of diet III was the same as diet II, except that it included 0.05 mg each of folic acid and biotin per 100 gm of ration.

<sup>2</sup> Cerelease.

guanidine or 2% succinylsulfathiazole was added to this diet at the expense of the glucose. The rats were then divided into 4 groups of 4 to 5 rats each, and supplemented with a single dose of *d*-calcium pantothenate, *dl*-ethyl 2'-monopalmitoxypantothenate (II), or *dl*-ethyl dipalmitoxypantothenate (I) equivalent to 500 µg of *d*-calcium pantothenate per rat. It has been reported that a single dose of calcium pantothenate at a level of 800 µg induces a marked weight gain in panto-

incidence of infestation with parasites which might produce iron-deficiency anemia.

Among the adults no residua clearly attributable to childhood rickets were noted and among the children no certain cases of clinically diagnosable rickets were seen. Serum phosphatase determinations were not done and no other laboratory or x-ray studies were undertaken. Breast feeding, the use of evaporated milk (which is fortified with vitamin D), the free exposure to the sun during much of the year, and the absence of a whole-cereal dietary contribute to this good situation relative to rickets.

Iodine lack as reflected by endemic goiter was not encountered to an extent to be considered of nutritional significance. Fluorosis is not severe in the population studied. Dental caries, however, was variable—in some geographic regions it was severe, in a few groups it was strikingly rare. In the examinees at Pinon, the region less influenced by white man's culture, the recorded incidence of noteworthy caries was slightly greater, but evidence of dental care (number of edentulous persons) was less. There was, in our opinion, no real difference between the dental status in these two regions, in contrast to that which one might expect from the widespread belief that the closer the food habits approach the primitive, the fewer dental ills.

Clinical evidence, vital statistics, and hospital experience reveal no widespread severe vitamin B-complex deficiency. There are no real problems of pellagra, of beriberi or of sprue. Such evidence is in accord with the qualitative dietary pattern. Oral lesions and conjunctival injection may speak for some incidence of ariboflavinosis, but no biochemical tests were made to decide this question. The dietary of some appears low in this nutrient, especially where milk is used in less quantity. It is suggestive that the incidence of these signs was greater at Pinon where the frequency of milk consumption was less. These observations lend support to the obvious need to increase the production, availability, and use of milk on the Reservation.

TABLE 2

Average weekly body weight gains of male rats on diets supplemented with various pantothenate preparations

Supple- ments <sup>2</sup>	Diet number	Vitamin level <sup>1</sup> Sulfate drug <sup>2</sup>					SST				
		20.0 µg		10.0 µg		7.0 µg		3.5 µg			
		SQ		SQ		---		SST		---	
		III		I		II		III		II	
Supple-	ments <sup>2</sup>	Period A		Period A		Period A		Period B		Period A	
		gm		gm		gm		gm		gm	
Calcium pantothenate		28.8 ± 3.0 <sup>4</sup>		17.6 ± 1.2		..		..		14.8 ± 2.4	
Ethyl dipalmitoxypantothenate		29.0 ± 1.5		20.8 ± 1.6		20.4 ± 1.8		..		12.4 ± 1.9	
Pantothenol		..		..		18.8 ± 1.7		26.6 ± 2.8		..	
Pantothenyl tripalmitate		..		..		18.8 ± 2.7		26.2 ± 2.1		..	

<sup>1</sup> The vitamin level is indicated as *D*-calcium pantothenate, micrograms per gram of ration.<sup>2</sup> SQ: Sulfaganidine, added at a level of 1% at the expense of the glucose. SST: Succinylsulfathiazole, added at a level of 2% at the expense of the glucose.<sup>3</sup> Period A: An assay period of three weeks following conditioning for two weeks. Period B: An assay period of three weeks following period A.<sup>4</sup> Standard error of the mean,  $\sqrt{2d^2/n(n-1)}$ .<sup>5</sup> Six rats were used for each of the groups supplemented with 20.0 µg of the vitamin supplement. In the rest of the groups, 5 rats were used for each group.

which we observed. These changes were sometimes of traumatic origin; more frequently, however, they were associated with evidence of an old trachomatous infection. History consistent with severe avitaminosis A was not obtainable in relation to these findings and the biochemical and other evidence fails to support a thesis that such impairment is attributable to malnutrition.

The serum cholesterol values among the Navajo fall into the usual pattern for omnivorous populations, and the levels found do not support the thesis that they may be responsible for a low incidence of coronary heart disease.

Since the initial impetus for this investigation was the exploration of a possible dietary influence on malignancy, it is appropriate to comment on this point. The nutritional level of the Navajo has not been found to differ markedly from that of several other populations which have been investigated. There is no pronounced peculiarity of the nutriture which in our present state of knowledge one might seize upon as a likely explanation of any true difference in malignancy rate which may exist between the Navajo and other groups. The extent of the present use of wild or native foods does not seem to us to justify the expectation that among these there is a likely explanation of the phenomenon. Further experimental studies of the effect of the particular diet as here described might conceivably reveal subtle unexpected effects, but in view of the well-recognized differences between racial groups in rate of incidence of cancer and other diseases, it may logically be hypothesized that a genetic influence is a more likely explanation than a dietary one. This conclusion is similar to that entertained concerning the low incidence of cervical cancer among Jewish women (Anonymous, '56).

This study is an initial step toward meeting the need expressed by Kraus ('54) for "scientifically conducted surveys of nutritional status among Indian populations of the Southwest..." It is hoped that it may be followed by additional investigations among this tribe and among the other Indian groups. We believe that information of this type made widely

cium pantothenate, ethyl monopalmitoxypantothenate or ethyl dipalmitoxypantothenate resulted in similar body weight gains (table 3). The gain in body weight reached a maximum at the 10th day. The growth response which was induced by calcium pantothenate after the first day appeared to be higher than the response observed in the other groups; the differences, however, were not statistically significant.

Esterification of the vitamin with palmitic acid increased the amount of pantothenic acid that appeared in the urine after the administration of a single large dose (table 4).

TABLE 4

*Average urinary excretion of pantothenate<sup>1</sup> per rat following the administration of a single large dose of various supplements*

SUPPLEMENTS <sup>2</sup>	HOURS AFTER ADMINISTRATION			TOTAL μg	% RECOVERY	AV. BODY WT. <sup>3</sup> gm
	0-12	12-24	24-36			
Calcium pantothenate	600	300	220	1120	11	203.3
Ethyl monopalmitoxy- pantothenate	1970	1030	260	3260	33	202.3
Ethyl dipalmitoxy- pantothenate	1270	800	370	2440	24	206.0

<sup>1</sup> The amounts are indicated as *d*-calcium pantothenate.

<sup>2</sup> Each rat was supplemented with a preparation at a level equivalent to 10 mg of *d*-calcium pantothenate.

<sup>3</sup> Each group consisted of three normal male rats. Analysis was made on a pooled sample from each group.

With calcium pantothenate, 11%, with the monopalmitate, 33% and with the dipalmitate, 24% of the pantothenic acid was recovered from the urine in 36 hours. In all of the three 12-hour collection periods, a higher excretion of pantothenic acid was observed after the palmitic acid esters were fed. During the first and the second periods supplementation with ethyl monopalmitoxypantothenate resulted in a higher excretion of the vitamin than was obtained with the dipalmitate. In the last period, however, the average excretion per rat fed the dipalmitate was higher than it was for those supple-



thenate was used as a supplement. The 71  $\mu$ g level of pantothenate, however, is within normal range for liver (Wright and Welch, '43, '44; Ford et al., '53; Everson et al., '54). During the course of the 12-hour experiment, it was noted that the amount of liver pantothenate tended to increase; the levels at the 6th and the 12th hour were 72  $\mu$ g and 82  $\mu$ g per gram of liver respectively. The results thus appeared to indicate that a gradual utilization of pantothenic acid took place when the dipalmitate of ethyl pantothenate was fed. However, a level of pantothenate equivalent to that of normal rat liver was noted within two hours after supplementation with the dipalmitate.

#### SUMMARY

The biological utilization of fat-soluble derivatives of pantothenic acid, ethyl dipalmitoxypantothenate and ethyl 2'-monopalmitoxypantothenate was compared with that of calcium pantothenate in rats. The over-all activity of the palmitic acid esters was equal to that of the water-soluble form as a supplement for pantothenic acid; this was proved by feeding experiments under various conditions. When a large single dose of the preparation was administered to the rats, the excretion of the pantothenic acid into the urine was markedly increased by esterifying the vitamin with one or two moles of palmitic acid. The biological utilization of the pantothenic acid moiety when present as an ester appeared to be slower than that of the free vitamin. The liver of pantothenate-deficient rats, however, contained a normal pantothenate level within two hours after the administration of ethyl dipalmitoxypantothenate. The activity of pantothenyl tripalmitate was found to be equal to that of free pantothenol.

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# THE BENEFICIAL EFFECT OF PROGESTERONE ON PREGNANCY IN THE VITAMIN A-DEFICIENT RABBIT<sup>1</sup>

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It is known that progesterone is required to maintain pregnancy in the rabbit (Allen and Corner, '29; Pincus and Werthessen, '38). It is also known that a deficiency of vitamin A causes an impairment in reproduction in the rabbit (Lamming, '49; Lamming et al., '54). The latter workers suggested that exogenous progesterone may alleviate some of the impairment in reproduction induced by a vitamin A deficiency.

This is a report of experiments in which an attempt was made to increase the reproductive performance of vitamin A-deficient female rabbits by the injection of progesterone.<sup>1</sup>

## EXPERIMENTAL

In the first experiment, 34 adult New Zealand white female rabbits were placed on a diet (Lamming et al., '54) containing no detectable carotene. After 4, 8 or 12 weeks on this diet, the females were mated to fertile males with one-half of the females receiving daily injections of 8 mg of progesterone in oil starting on the day of mating. This dosage of progesterone was selected to make it well in excess of the 4 mg minimum daily level required to maintain pregnancy in the rabbit (Allen and Heckel, '39). At the conclusion of this experiment the

<sup>1</sup>An abstract of this paper was presented at the 47th Annual Meeting of the American Society of Animal Production 1954.

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jections enabled the females to carry the young for a much longer period of time.

The low number of corpora lutea in the untreated groups on the diet for 12 or 13 weeks was probably due to abortion or resorption early in gestation which has been shown to occur in rabbits on a carotene-deficient diet (Lamming et al., '54). If pregnancy is terminated in the early stages, the corpora lutea are not grossly discernible 28 days post coitum.

TABLE 1

*The reproductive performance of New Zealand white female rabbits on a carotene-deficient diet*

NO. OF FEMALES	WKS. ON DIET BEFORE MATING	TREATMENT	AV. YOUNG/FEMALE			AV. C.L.	AV. LIVER VIT. A	AV. PLASMA VIT. A
			Living	Dead	Sites		$\mu\text{g}/\text{gm}$	$\mu\text{g}/100\text{ ml}$
6	4	None	5.0	1.8	0.8	9.6	9.9	8.2
6	4	Prog. <sup>1</sup>	3.7	0.3	0.5	7.2	8.3	8.0
5	8	None	4.0	0.6	0.0	7.6	2.8	3.6
5	8	Prog. <sup>1</sup>	2.5	1.3	0.5	9.0	1.0	6.4
6	12	None	0.8	0.8	0.0	1.0	1.8	7.6
6	12	Prog. <sup>1</sup>	4.3	2.2	0.3	9.8	2.0	9.9
8	13	None	1.0	0.5	2.1	5.0	0.5	2.0
8	13	Prog. <sup>2</sup>	5.1	3.4	0.0	10.2	0.5	2.5
8	13	Vit. A <sup>3</sup>	1.2	2.9	1.0	5.5	787.0	40.7

<sup>1</sup> Daily injection of 8 mg.

<sup>2</sup> Daily injection of 12.5 mg.

<sup>3</sup> Weekly feeding of 100,000 I.U. vitamin A acetate.

As a result of this, the number of corpora lutea 28 days post coitum was not an accurate measure of the number of ovulations.

Since the levels of vitamin A found in table 1 were taken 28 days post coitum, they were undoubtedly higher at the beginning of pregnancy. It is not known whether the levels of vitamin A were sufficiently low during pregnancy in the 4- and 8-week groups to cause a decrease in the number of live young. However, feeding this carotene-deficient ration for 12 weeks caused a marked reduction in reproductive efficiency.



12 or 13 weeks before mating averaged 0.9 living and 0.6 dead young at autopsy on the 28th day of pregnancy, while 7 comparable females injected with 8 mg of progesterone daily had 4.3 living and 2.2 dead young and the 7 females receiving 12.5 mg of progesterone had 5.1 living and 3.4 dead young.

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Food intake and weight gain were measured for a period of 20 days. As the experimental situation demanded, this ration was supplied either ad libitum or in restricted quantities. In one restriction experiment a nitrogen-free supplement (298, table 1) was offered ad libitum. All the animals were housed individually in screen-bottom metal cages maintained in an air-conditioned animal room.

TABLE 1  
*Percentage composition of diets*

INGREDIENT	DIET			
	287 <sup>1</sup>	292 <sup>1</sup>	298	311 <sup>1</sup>
Casein <sup>2</sup>	5	5	..	..
Sucrose	..	..	45	..
Dextrin	85	..	..	70
Starch	..	85	..	..
Salts A <sup>3</sup>	5	5	5	..
Salts HMW <sup>2</sup>	..	..	..	5
Corn oil	5	5	5	5
Hydrogenated vegetable oil <sup>4</sup>	..	..	45	20

<sup>1</sup> Vitamin supplement and composition of Salts A as reported by Fenton and Carr ('51).

<sup>2</sup> Labco, vitamin-free.

<sup>3</sup> Hubbell, Mendel and Wakeman ('37).

<sup>4</sup> Crisco.

The protein minima studies followed the principles outlined by Melnick and Cowgill ('37). Adult mice of the A/Fn and I/Fn strains were maintained in individual metabolism cages. These animals had been reared to age 6 months on a commercial stock ration.<sup>2</sup> The mice were fed a nitrogen-free diet (311, table 1) ad libitum. Urine collections were made during the third and 4th days on this diet. During the following three days 5 mg of nitrogen were injected intraperitoneally daily in the form of an aqueous solution of enzymatic casein hydrolyzate. During a second and third three-day period the amount of nitrogen was increased to 10 and 15 mg per day respectively.

<sup>2</sup> Purina Laboratory Chow.

# THE BIOLOGICAL VALUE OF OILS AND FATS

## IV. THE RATE OF INTESTINAL ABSORPTION

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### INTRODUCTION

Earlier experiments established that the increase in weight of young rats is dependent on the type of the dietary fat (Thomasson, '55a). These investigations further revealed that the different growth-action of the fats and oils investigated is actually due to a different food intake.

It is conceivable that food intake and growth are correlated with the rate at which the fat in question is absorbed. The literature in this field is very restricted, however. Steenbock et al. ('36) established that halibut- and cod-liver oil are absorbed significantly more rapidly than lard and maize oil. In addition, a number of fats and oils could be arranged in the order of decreasing rates of absorption as follows: linseed oil, olive oil, whale oil, soya-bean oil, groundnut oil, rancid lard, cottonseed oil, coconut fat, and palm oil. Deuel et al. ('40) observed no differences in the rates of absorption of cottonseed oil, butterfat, and coconut fat; they found, however, that rapeseed oil was absorbed at a much lower rate. In later investigations carried out in Deuel's laboratory it could be shown that maize oil (Bavetta and Deuel, '42) and lard (Crockett and Deuel, '47) possessed the same rate of absorption. In addition, Bhalarao et al. ('47) found that sesame oil, coconut fat, and butterfat are absorbed at the same rate, and safflower, groundnut and cottonseed oil slightly, although not significantly, slower.



(43 gm/20 days), they actually lost weight. When other mice of the C3H and A strains were similarly restricted but were allowed in addition unlimited quantities of the nitrogen-free ration 298, they again showed positive weight gains. We have omitted data of those animals which did not completely consume the 43 gm of diet 287. The sum of diet 287 and diet 298 consumed did not equal the quantity of diet 287 consumed under conditions of ad libitum feeding (table 2).

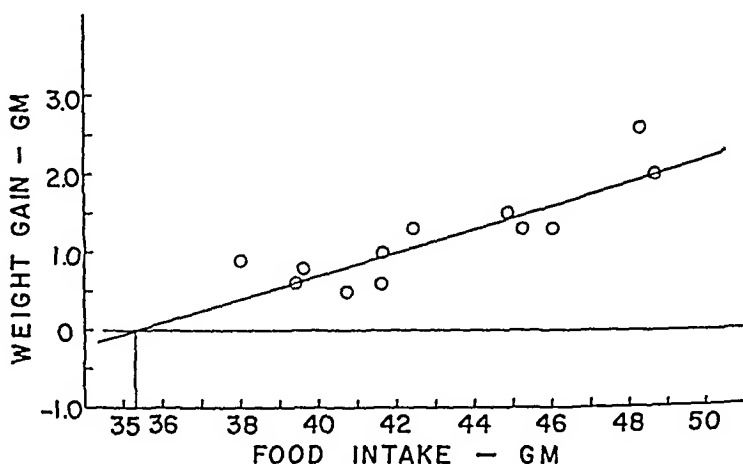


Fig. 1 Regression line of weight gain/20 days on voluntary intake of 5% protein diet of weanling C57BL male mice. Zero weight gain theoretically at food intake of 35.3 gm.

In figure 1 are plotted the body weight gains of individual C57BL animals against food intake under conditions of ad libitum feeding. The calculated regression line shows that weanling mice of this strain should show no weight change if consuming 35.3 gm of diet 287/20 days. As a check 15 weanling male mice of the C57BL strain were fed this amount of diet 287 over a period of 20 days. The average weight gain observed for the entire group was 0.1 gm. On the other hand, as was shown in table 2, mice of the A strain lost weight even when fed as much as 43 gm of diet 287. In a further experiment mice of the A strain were each fed 36 gm of diet 287 mixed with 18 gm of dextrin. The resulting mixture supplied approx-

spectively after administering the fat. From these values it was calculated at what time 50% of the dosage would have disappeared from the gastrointestinal tract. The value so obtained (it varied from 5 to 11 hours) was applied to a third series of 6 animals.

When calculating  $AT_{50}$  it was supposed that, at any rate within the scope of the relevant observations, a linear relationship exists between time and the percentage of fat absorbed.  $AT_{50}$  was calculated as follows:  $AT_{50} = \bar{x} + \frac{50 - \bar{y}}{b}$ ,

in which

$x$  = absorption time = time interval between administering the fat and the analysis (in mins.);

$y$  = percentage of fat recovered in the gastrointestinal tract after absorption time  $x$ ;

$\bar{x}$  = the mean of all  $x$  values;

$\bar{y}$  = the mean of all  $y$  values;

$$b = \text{regression-coefficient of } y \text{ upon } x = \frac{\sum xy - \frac{\sum x \cdot \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}},$$

in which  $n$  = number of observations.

The 95% confidence interval of the  $AT_{50}$  value is:

$$\bar{x} + c \cdot \frac{50 - \bar{y}}{b} \pm t_{(n-2)} \cdot s_{x_{50}},$$

in which

$s_{x_{50}}$  = standard deviation of the  $AT_{50}$ -value;

$t_{(n-2)}$  = critical value from Student-distribution for  $P_2 = 0.05$ ;

$c$  = correction factor for skewness =  $b^2 \cdot (b^2 - t_{(n-2)}^2 \cdot s_b^2)$ , in which  $s_b$  = standard deviation of the regression coefficient.

In the calculation of  $AT_{50}$  the values for the entire gastrointestinal tract have been employed. Actually, however, separate data for each of the three parts — stomach, small and large intestine — are available.

## RESULTS AND DISCUSSION

The amounts of fat recovered in the entire gastrointestinal tract after administration of 400 mg per 100 cm<sup>2</sup> of body surface are shown in table 1 as percentages of the dosage. These

When diet 292 was fed to weanling C57BL males, food intake amounted to only 32.4 gm, and a weight loss of 0.4 gm was observed over the 20-day period. This is precisely the weight loss predicted from the regression line in figure 1. Diet 292 is identical with diet 287 except that starch was substituted for dextrin.

The results of the protein minima studies are shown in figure 2. The endogenous urinary nitrogen excretion of I strain animals was significantly greater than that of A strain mice. At all levels of nitrogen intake the I strain mice showed more negative nitrogen balances.

#### DISCUSSION

The large strain differences in food intake under conditions of ad libitum feeding are in line with observations we have made with other diets. Under most feeding conditions the voluntary food intake of A and C3H mice has been found to exceed that of C57BL and I strain animals. It is the A and C3H strain which can most readily be made obese by nutritional means. The observation that mice of these two strains lose weight when restricted to the quantity of food voluntarily consumed by C57BL and I strain animals indicates that they not only voluntarily consume more food, but that they also require more food in order to show gains in body weight on low protein diets. With the diets fed in these experiments the question is raised whether the A and C3H mice require more protein in order to grow or whether the need is for additional calories. The positive weight gains observed when the low protein diet was fed in restricted amounts but the nitrogen-free ration supplied in unlimited quantities indicates that the need is for calories alone. This is further supported by the experiment in which A strain mice were fed 36 gm of the low protein diet mixed with 18 gm of dextrin. It may be concluded then that A and C3H mice have a greater need for calories than do C57BL and I strain animals for the utilization of low protein diets.

In table 1 the  $AT_{50}$  values (= number of minutes after which 50% of the fat administered disappears from the gastrointestinal tract) calculated from these percentages, are also recorded. In addition, values are given for the 95%-interval representing the limits between which lie 95% of the  $AT_{50}$  values of the respective fats. From these statistical data the fats under investigation can be arranged in 5 classes of decreasing rates of absorption, although it is not certain whether sesame oil and lard belong to group II or to group III or form a separate group. The influence of these oils and fats on the increase in weight of new-born male rats is known from previous investigations (Thomasson, '55a). On the basis of these findings the fats have been arranged according to decreasing growth-action; these rank numbers are given in the last column of table 1. It appears that a correlation exists between the rate of absorption ( $AT_{50}$  values) and the growth-action ( $r = 0.62$  with  $P < 0.01$ ).<sup>1</sup>

Such a correlation suggests that the divergent growth-action of various fats and oils [which, as previously shown (Thomasson, '55), is in turn determined by the food intake], might be due to the rate at which these fats are absorbed. Some doubt regarding this supposition is justified, however, as certain fats do not conform to it. The growth-action of lard, shea butter, olive oil and poppyseed oil for example, is too favorable in respect of their relative slow rate of absorption, whereas coconut fat and maize oil have a poorer growth-action than would be expected from the favorable rates of absorption of these fats. However, rapid growth and a correspondingly rapid absorption need not necessarily be considered as favorable. It has been shown (Thomasson, '55b) that longevity on butterfat-containing diets is less than when this fat is replaced by rapeseed oil, in spite of the fact that with the former fat the growth rate and the rate of absorption

<sup>1</sup> This correlation was tested by numbering the fats investigated according to decreasing growth-promoting ability and according to decreasing rate of absorption, and applying the method of rank correlation to these two series of values (Dixon and Massey, '51).

the conditions of our experiments, accumulation of excess fat without the deposition of additional protein.

#### SUMMARY

Two strains of mice highly susceptible to nutritionally induced obesity were found to have a higher caloric requirement for the utilization of a low protein diet than a strain which is moderately susceptible and one that is completely resistant. One obesity-susceptible strain required less nitrogen than the resistant strain.

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The mean values after three, 6 and d hours are 23.0, 21.9 and 22.7% respectively. This homogeneity prompted the calculation of the mean percentage from all the 56 observations: 22.5% with a standard error of 0.945%. Thus, this value

TABLE 2  
*Percentage distribution, over stomach, small and large intestine,  
of the recovered fat*

TYPE OF FAT	DURATION OF ABSORPTION: 3 HOURS			DURATION OF ABSORPTION: 6 HOURS			DURATION OF ABSORPTION: D HOURS <sup>1</sup>			d	
	stomach	intestine		stomach	intestine		stomach	intestine			
		small	large		small	large		small	large		
Butterfat	74	25	2	77	21	2	58	35	7	5	
Maize oil	78	20	2	65	27	8	66	32	2	6	
Cottonseed oil	84	16	0	74	24	2	68	28	4	6	
Beef tallow	85	14	1	85	15	0	87	10	3	6	
Coconut fat	74	22	4	75	19	6	63	27	10	6	
Soya-bean oil	62	33	5	58	38	4	66	28	6	6	
Sunflower oil	81	17	2	77	19	4	74	23	3	6	
Groundnut oil	69	31	0	78	20	2	64	29	7	6	
Olive oil	67	31	2	72	23	5	79	16	5	6	
Sesame oil	70	29	1	67	29	4	77	20	3	6	
Lard	78	22	0	73	21	6	82	14	4	6	
Palm fat	87	10	3	77	19	4	{	58	32	10	8½
								71	19	10	7
Whale oil	72	25	3	76	21	3	72	21	7	7½	
Shea butter	66	29	5	60	22	18	50	35	15	9½	
Herring oil	71	29	0	60	34	6	71	25	4	8	
Rapeseed oil	70	23	7	75	18	7	{	73	15	12	8
								58	19	23	9
Poppyseed oil	76	22	2	80	14	6	85	11	4	11	
Kapokseed oil	84	16	0	86	10	4	78	15	7	10½	
Mean	74.0	23.0	2.2	73.1	21.9	5.1	70.0	22.7	7.3		

<sup>1</sup> Time at which, according to an estimation based upon the absorption values after three and 6 hours, 50% of the fat would have been absorbed.

seems to be independent of the time of absorption and, independent, therefore of the total amount of fat present in the gastrointestinal tract. Probably the body has a mechanism at its disposal which attempts to maintain the percentage of fat in the small intestine at a constant level, namely 22.5% of

cellular migration in inflammation; (4) capillary permeability, as measured by the Menkin dye accumulation technique; and (5) cellular composition of bone marrow. A considerable decrease of complement activity and a substantial reduction in cellular migration to an inflamed area were observed in the white rats maintained on the niacin-tryptophan-deficient diet and the pyridoxine-deficient diet. Although a significant reduction in cellular migration occurred in deficient animals, no alteration in capillary permeability could be measured by the dye accumulation technique (Menkin, '40).

Although much work has been done with vitamin B<sub>12</sub> and its hematopoietic activity, very little research has been reported concerning its influence on resistance to disease. Neither have studies of this nature been performed on white rats maintained on a folic acid-deficient diet. The purpose of the previous investigations (Wertman et al., '53, '54, '55) was to study the various nonspecific physiological factors of resistance to infection concurrently in animals maintained on a well-defined diet that was deficient in thiamine, niacin-tryptophan and pyridoxine. The purpose of this investigation was to perform identical studies with a group of white rats maintained on a well-defined diet that was deficient in vitamin B<sub>12</sub> as well as a similar group maintained on a well-defined diet deficient in folic acid.

#### EXPERIMENTAL

Male, weanling albino rats of the Sprague-Dawley strain, approximately 21 days old, were employed in the investigation. All rats were housed individually in wide-mesh, screen bottom metal cages. The animals for the vitamin B<sub>12</sub> experiments were arranged into three groups, i.e., vitamin B<sub>12</sub> deficient, inanition controls and ad libitum controls. The deficient group contained 80 animals and the two control groups each contained 40 animals. All animals were observed and weighed daily.

these oils and fats could be divided into 5 groups according to a decreasing rate of absorption:

1. Butterfat.
2. Maize oil, cottonseed oil, beef tallow, coconut fat, soya-bean oil, sunflower oil, groundnut oil and olive oil.
3. Sesame oil, lard, palm fat and whale oil.
4. Shea butter and herring oil.
5. Rapeseed oil, poppyseed oil and kapokseed oil.

A significant correlation appeared to exist between the rate of absorption and the growth-action of the oils and fats.

The fat recovered three to 11 hours after the administration of 400 mg per 100 cm<sup>2</sup> of body surface appeared to be distributed over stomach, small and large intestine with a certain constancy, averaging 73, 22.5 and 5% respectively.

The percentage of fat in the small intestine, seems to be independent of the absorption time and consequently of the amount of fat in the tract; that in the stomach showed a tendency to decrease while that in the large intestine increased when absorption time was extended.

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mixture no. 2,<sup>2</sup> 4.00; corn oil,<sup>3</sup> 2.00; sulfasuxadine, 1.00; choline chloride, 0.20; i-inositol, 0.03; *dl*-alpha-tocopherol acetate, 0.01; and 2-methyl-1,4-naphthoquinone, 0.001.

Each animal in the folic acid studies received a vitamin pill daily. The pills prepared for the control animals contained the following vitamins in micrograms: thiamine, 40; riboflavin, 60; pyridoxine, 50; calcium pantothenate, 300; nicotinic acid, 150; biotin, 3; and folic acid, 20. Lactose was used as the binder in the preparation of the pills. Folic acid was omitted from the pills prepared for the vitamin-deficient group of animals.

TABLE 1  
*Distribution and initial and final mean weights of rats*

GROUP	NUMBER OF RATS	VITAMIN B <sub>12</sub> DEFICIENCY		NUMBER OF RATS	FOLIC ACID DEFICIENCY	
		Mean body weights			Mean body weights	
		Initial	Final		Initial	Final
		gm	gm		gm	gm
Ad libitum controls	40	58.2	176.8	10	43.1	203.9
Inanition controls	40	59.4	115.5	14	43.6	126.2
Deficient	80	56.7	112.7	39	43.5	125.5

The addition of sulfasuxidine to a diet has been reported to have an adverse effect on the bacterial synthesis of biotin in the rat intestine (Martin, '42; Welch, '42). A biotin deficiency is produced as well as interference with the utilization of pantothenic acid (Wright and Welch, '44). It was for these reasons that a supplement of biotin, 2 µg, and calcium pantothenate, 150 µg, was added beyond the accepted normal requirement of the rat.

In addition to the vitamins supplied in the basal diet and the supplementary pills, each animal received 3000 USP units of vitamin A and 24 USP units of vitamin D each week.<sup>7</sup> The animals were maintained on the basal diet and vitamin preparations for a period of 36 days. In the last few days of this period, a high death rate became apparent. Initial and

<sup>7</sup> See footnote 6, page 475.

AMINO ACID IMBALANCE  
AS RELATED TO METHIONINE, ISOLEUCINE,  
THREONINE AND TRYPTOPHAN  
REQUIREMENT OF THE  
RAT OR MOUSE<sup>1</sup>

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Recent reports from this laboratory by Salmon ('54) and by Sauberlich and Salmon ('55) have been concerned with the production of an amino acid imbalance in the rat. The reduction in growth due to this imbalance could be corrected by dietary supplements of tryptophan, but not by niacin alone. From these studies it was revealed that the tryptophan requirement of the rat is not a constant factor, but is related to the diet employed and in particular to the protein or nitrogen level of the diet. Tryptophan is peculiar among amino acids because of its relationship to niacin. Therefore, it was of interest to determine whether or not similar imbalance conditions with other amino acids could be produced.

The present report is concerned with the production of methionine, isoleucine and threonine imbalances in the rat.

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final mean weights for each group in the folic acid experiments appear in table 1.

The day before the rats were to be sacrificed, blood samples for complete blood counts were obtained from all animals by tail bleeding. Standard hematological techniques were employed for these counts. The results are recorded in table 2. Following the tail bleeding, each rat was injected intraperi-

TABLE 3

*Complement activity of vitamin B<sub>12</sub>- and folic acid-deficient and control rats*

GROUP	SERUM POOL <sup>2</sup>	VITAMIN B <sub>12</sub> COMPLEMENT ACTIVITY E. U. <sup>1</sup>	SERUM POOL	FOLIC ACID COMPLEMENT ACTIVITY E. U. <sup>2</sup>
Ad libitum controls	1	0.08 ml of 1:6 dilution	1	0.12 ml of 1:6 dilution
	2-5	0.12 ml of 1:6 dilution	2-5	0.15 ml of 1:6 dilution
	6	0.10 ml of 1:6 dilution		
	7-8	0.14 ml of 1:6 dilution		
Inanition controls	1-2	0.12 ml of 1:6 dilution	1-3	0.21 ml of 1:6 dilution
	3-4	0.20 ml of 1:6 dilution	4	0.24 ml of 1:6 dilution
	5-8	0.14 ml of 1:6 dilution		
Deficient animals	1	0.16 ml of 1:6 dilution	1	0.24 ml of 1:6 dilution
	2-3	0.14 ml of 1:6 dilution	2-3	0.21 ml of 1:6 dilution
	4	0.18 ml of 1:6 dilution	4	0.24 ml of 1:6 dilution
	5-6	0.14 ml of 1:6 dilution	5-8	0.21 ml of 1:6 dilution
	7	0.16 ml of 1:6 dilution	9	0.18 ml of 1:6 dilution
	8-9	0.14 ml of 1:6 dilution		
	10	0.16 ml of 1:6 dilution		
	11-16	0.12 ml of 1:6 dilution		

<sup>1</sup> One exact unit.

<sup>2</sup> Five rats were used in each pool.

toneally with 10 ml of inflammation-iciting fluid. This fluid was a mixture of sterile Locke's solution and double strength nutrient broth in the ratio of 85 to 15 on a volume basis.

Twelve hours after the intraperitoneal injections, the rats were anesthetized with ether and bled to death by the cardiac puncture technique. The blood specimens so obtained were allowed to clot and the sera were collected, pooled, and utilized for the determination of complement activity as described by Wertman, Smith and O'Leary ('54). The results of these



TABLE 4  
Total and differential leucocyte count of peritoneal exudates in vitamin B<sub>12</sub> and folic acid-deficient and control rats

EXUDATE CELLS	AD LITHIUM CONTROL	INANITION CONTROL	VITAMIN B <sub>12</sub> DEFICIENT	AD LITHIUM CONTROL	INANITION CONTROL	FOLIC ACID DEFICIENT
Total leucocytes, cells/mm ( $1 \times 10^7$ )	Median M.D. <sup>1</sup> Range	22.4 1.87 20.2-26.9	16.6 1.68 13.1-19.6	17.8 3.2 14.2-26.8	19.8 2.7 0.5-5.3	2.8 1.0 10.3-30.4
Granulocytes, %	Median M.D. Range	21.5 6.0 8.5-43	13.4 4.4 4.5-30	37.5 4.0 28-43	41.0 4.0 34-50	11.5 2.7 4-17
Lymphocytes, %	Median M.D. Range	74.3 4.5 53-89	82.2 5.1 66-94.5	28.5 2.9 22-33	17.5 2.8 13-24	25 3.2 17-32
Monocytes, %	Median M.D. Range	4.8 0.7 2-10.5	4.4 1.2 0.5-8	35 4.4 26-45	40 4.3 30-48	65 5.1 52-76

<sup>1</sup> Mean deviation.

gained in weight an average of 34.0 gm per week during the 4-week experimental period. However, when 20% of oxidized casein was added to the diet and supplemented with 0.2% of DL-tryptophan, the growth of the animals was reduced to 17.1 gm per week (table 2, group 2). The methionine imbalance also produced a marked increase in the amount of food consumed per gram of gain of the animals, as may be noted from the ratios of food consumption to body-weight gain.

Supplements of either methionine or vitamin B<sub>12</sub> partly reversed the reduction in growth while a supplement of both methionine and vitamin B<sub>12</sub> gave essentially normal growth and food conversion (31.7 gm per week). Supplements of vitamin B<sub>12</sub> or methionine also improved somewhat the growth of rats fed the 42% peanut basal diet.

In experiment B (table 2, groups 9 to 12), the level of peanut meal used in the diet was reduced to 35% to restrict the methionine content more rigidly. The diet was supplemented with lysine, cystine, folacin and vitamin B<sub>12</sub> (table 1). This diet permitted the same growth as that obtained with the 42% peanut meal diet unsupplemented with vitamin B<sub>12</sub>. Again the addition of 20% oxidized casein to the 35% peanut meal diet caused a marked reduction in growth and rate of food conversion of the animals despite the presence of vitamin B<sub>12</sub> in the diet (36.5 to 19.7 gm per week, respectively). Supplementation of the diet with 0.5% of DL-methionine almost completely corrected the imbalance (30.1 gm per week), whereas, the addition of aureomycin to the imbalance diet had little, if any, effect.

An amino acid imbalance of methionine was also readily produced in weanling rats of the AES strain when fed the diets employed in the above experiments. The imbalance condition was also corrected by methionine. Since the results were essentially the same as those obtained with the SD strain of rats, data were not presented. These experiments also revealed that rats of the SD strain were more efficient than rats of the AES strain in the conversion of food to gains

was reduced in vitamin B<sub>12</sub>-deficient animals as compared to ad libitum controls. Inanition produced a slight decrease in the cellular migration but not nearly as great as the decrease in deficient animals. Differential counts of the peri-

TABLE 5

*Cellular composition of bone marrow in vitamin B<sub>12</sub>-deficient and control rats*

BONE MARROW CELLS		DIET		
		Ad libitum	Inanition	Vitamin B <sub>12</sub> deficient
Total granulocytes, %	Median	35.2	37.3	33.5
	Range	22.6-45.5	26.6-49.6	20.6-45.9
Promyelocytes and myelocytes, %	Median	9.6	10.5	9.2
	Range	7-13.6	8-14	6-12.6
Metamyelocytes and segmenters, %	Median	22.3	23	21.4
	Range	14.3-27.6	17-28.3	13.3-27.3
Eosinophiles, %	Median	3.3	3.8	2.9
	Range	1.3-4.3	1.6-7.3	1.3-6
Nucleated red blood cells, %	Median	51.3	48.4	52.8
	Range	45-64	41.3-60.3	45.3-66
Lymphocytes, %	Median	8.9	9.8	8.3
	Range	6.3-12.6	8-12.6	5.3-12.6
Monocytes, %	Median	1.8	2.4	1.6
	Range	1-3.2	1-4	0.6-3
Blast cells, %	Median	0.9	0.5	0.8
	Range	0-7.3	0-2.3	0.7
Mast cells, %	Median	0.5	0.7	0.4
	Range	0-1.9	0-1.6	0-1.9
Plasma cells, %	Median	0.3	0.2	0.4
	Range	0-1.6	0-1.9	0-1.6
Unclassified, %	Median	0.2	0.4	0.2
	Range	0-1.3	0-1.3	0-1.3

toneal exudate revealed a lymphocytosis with a concomitant granulocytopenia. The granulocytes are the most active in phagocytosis (Menkin, '40, '50), so it appears that the resistance of the deficient animals might be reduced in this instance.

(Sauberlich et al., '53). Therefore, the basal diets were supplemented with these amino acids. Isoleucine was added in amounts sufficient to provide only a suboptimal level (table 1, diets 5 to 8). Hemoglobin, because of its low isoleucine content, was employed in an attempt to produce an imbalance condition.

From the results summarized in table 3, it may be noted that the addition of hemoglobin to the corn basal diet caused a very severe inhibition in growth. In experiment C, growth was reduced from an average gain of 8.7 gm per week to only 0.9 gm by the addition of 15% hemoglobin to the 75% basal corn diet (table 3, groups 1 and 2). Supplementation of the diet with 0.55% of DL-isoleucine readily reversed the depression in growth.

In experiment D (table 3), the content of corn in the basal diet was reduced to 70%, but the level of DL-isoleucine was increased to 0.2% to permit increased growth of the animals. However, when 20% of hemoglobin was added to the diet, a severe inhibition in growth was again noted (16.3 vs 1.2 gm per week, respectively). Deaths were noted when the animals were maintained on the imbalance diet for periods beyond 4 weeks. An increased addition of DL-isoleucine (0.4%) to the diet prevented death and depression in growth. The amount of food required per gram of gain in body weight was also markedly increased, but was returned to normal with an increased supplementation of isoleucine to the diet.

#### *Effect of threonine imbalance on growth of the rat*

When a series of amino acids was added to a 6% casein-corn grits basal diet (table 1, diets 9 and 10), growth of weanling rats was very markedly reduced. For example, in experiment E (table 4, groups 1 to 3), growth of the rats fed the 6% casein-corn grits basal diet was reduced from an average weekly gain of 19.6 gm to only 7.3 gm by the addition of the amino acids to the diet. However, the depression in growth was prevented by supplementation of the diet with



the ad libitum-control rat exudate. The mononuclear leucocyte count of peritoneal exudates in folic acid-deficient rats was 25% greater than in the inanition controls and 30% greater than in the ad libitum-control animals.

Experiments were performed to determine whether or not "leukotaxine" activity was present in vitamin B<sub>12</sub>-deficient, folic acid-deficient and control animals. No alteration in capillary permeability could be detected by the Menkin dye accumulation technique. In every instance, the cell-free peritoneal exudate from all groups of animals produced the same type and degree of reaction in the skin of the rabbit after injection of dye. These results are in complete agreement with those of the three previous papers in this series (Wertman et al., '53, '54, and '55).

No significant difference in the cellular composition of the bone marrow of the vitamin B<sub>12</sub>-deficient, inanition-control and ad libitum-control rats was noted. These results correspond with those obtained by Wang, Scheid and Schweigert ('54) in their experiment using varying levels of vitamin B<sub>12</sub>. No marked changes were found in the bone marrow composition of the vitamin B<sub>12</sub>-deficient rats as compared to the normal rats (table 5).

The bone marrow preparations of the folic acid-deficient rats exhibited a relative decrease in granulocytes as compared to the ad libitum and inanition controls. This decrease was caused primarily by a severe decrease in the percentage of older forms of the granulocytic elements, such as metamyelocytes and segmenters. There was a significant decrease in the percentage of lymphocytes in the marrow of the deficient and inanition-control animals. Nucleated erythrocytes in the marrow of the deficient rats decreased 7.0% over the ad libitum controls, and 8.5% over the inanition controls. An increase in reticulocytes was also noted in the bone marrow of folic acid-deficient rats. These were placed in the "unclassified" group (table 6).

TABLE 4

*Effect of threonine on the growth of weanling rats fed threonine imbalance diets*  
(SD rats; 4-week experimental period)

GROUP NO.	BASAL DIET NO.	DIET	NO. OF RATS	AV. GAIN/ EAT/WK.	AV. DAILY FOOD CON- SUMPTION/ EAT	GM FOOD INGESTED GM GAIN
				gm	gm	
EXPERIMENT E						
1	9	6% Casein	5	19.6	9.0	3.1
2	10	6% Casein + amino acids	6	7.3	3.8	3.7
3	10	Same as group 2, + threonine <sup>1</sup>	5	18.0	5.9	2.3
4	10	Same as group 3, lysine omitted	5	19.0	8.5	3.1
5	10	Same as group 3, histidine omitted	5	22.5	7.4	2.3
6	10	Same as group 3, isoleucine omitted	5	18.0	6.4	2.5
7	10	Same as group 3, leucine omitted	5	24.3	7.8	2.3
EXPERIMENT F						
8	9	6% Casein	4	18.4	7.9	3.0
9	9	6% Casein + T + M <sup>2</sup>	4	18.2	8.1	3.1
10	11	6% Casein + amino acids	5	10.4	6.8	4.6
11	11	Same as group 10, + threonine	4	23.8	8.3	2.4
EXPERIMENT G						
12	12	10% Casein	4	22.4	9.1	2.8
13	13	10% Casein + amino acids	4	12.9	7.2	3.9
14	13	Same as group 13, + threonine	3	22.6	8.6	2.7

<sup>1</sup> DL-Threonine, supplemented at a level of 4 gm/kg diet.

<sup>2</sup> T = DL-tryptophan, 1 gm/kg diet; M = DL-methionine, 3 gm/kg diet.

4. The vitamin B<sub>12</sub> deficiency appeared to have a marked effect upon the types of leucocytes migrating to the site of inflammation. There was a marked lymphocytosis and granulocytopenia in the exudates of the vitamin B<sub>12</sub>-deficient rats. The inflammatory exudates of the folic acid-deficient rats showed a relative decrease in number of polymorphonuclear leucocytes and a relative increase of mononuclear leucocytes.

5. No alteration in capillary permeability, as measured by Menkin dye accumulation technique, was noted in either vitamin B<sub>12</sub>- or folic acid-deficient rats.

6. No significant change was observed in the cellular composition of the bone marrow of the vitamin B<sub>12</sub>-deficient or inanition-control animals as compared to the ad libitum-control rats.

7. The bone marrow preparations from the tibias of the inanition and folic acid-deficient rats presented a relative decrease of lymphocytes. A severe decrease in relative numbers of metamyelocytes and segmenters, a slight decrease in promyelocytes and myelocytes, and an increase of reticulocytes were noted in the bone marrow removed from the tibias of folic acid-deficient rats.

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by the animal to remove the excess unused amino acids. In so doing, tryptophan was also lost.

In an attempt to determine further the effect excess amino acids may have on the imbalance phenomenon, adult rats were placed on a protein-free diet (table 1, diet 14). Other groups of animals received this diet supplemented with proteins deficient in certain amino acids, or with glycine, or with urea. Under these more pronounced conditions, one could then, perhaps, determine whether or not excess amino acids exerted an effect by causing an increased urinary loss of amino acids limiting to the animal. Such losses of amino acids may then be reflected in an increased rate in loss of body weight and a reduced survival time of the animals.

Results of such a study are summarized in table 5. The data indicate that rats fed the diets supplemented with amino acid-deficient proteins, glycine or urea lost weight at no greater rate than animals fed the protein-free diet. Similarly, the excess amino acids or nitrogen did not reduce the survival time of the animals. The hemoglobin values of all animals remained near normal for a period of at least 11 weeks, even with the complete absence of protein in the diet. Food consumption of the animals also remained surprisingly high throughout the experiment.

*Plasma amino acid levels of rats fed  
certain imbalance diets*

Summarized in table 6 are levels of several amino acids in the plasma of rats fed certain imbalance diets. In experiment I, rats were fed the methionine-imbalance diets. From these experiments, it was noted that the methionine level in the plasma was not affected by the addition of 20% oxidized casein to the diet. The supplementation of the peanut meal-oxidized casein diet with 0.5% of DL-methionine increased the level of methionine from 10.6 to 20.6  $\mu\text{g/ml}$  of plasma (table 6, groups 2 and 3). Only the L-isomer of methionine was measured in the microbiological method employed. The ad-



dition of vitamin B<sub>12</sub> to the imbalance diet increased the plasma level of methionine to 15.2 µg/ml. Supplementation of the basal peanut meal diet with methionine likewise produced a very marked increase in the concentration of methionine in the plasma (10.4 to 18.6 µg/ml). Vitamin B<sub>12</sub> supplementation also produced some increase.

The valine level in the plasma, however, was increased nearly three-fold by the addition of oxidized casein to the basal peanut meal diet (8.4 to 24.6 µg/ml). Supplements of methionine or vitamin B<sub>12</sub> to the diets had little effect on the level of valine in the plasma.

In experiment J, the methionine, proline, tryptophan and valine levels were determined in plasma from adult rats fed a 20% normal casein diet or a 20% oxidized casein diet supplemented with both tryptophan and methionine or only with methionine for a period of three weeks. The plasma amino acid levels of rats fed the casein diet or the fully supplemented oxidized casein diet were essentially the same, except for methionine. The methionine level was somewhat higher for rats fed the casein diet. This may be related to the level of DL-methionine added to the oxidized casein diet.

When tryptophan was omitted from the diet, the plasma amino acid levels were considerably lowered. This occurred despite the fact that the animals were consuming considerable quantities of amino acids, in addition to losing in body weight an average of 24 gm per week. Thus, the animals apparently were capable of removing from the blood stream considerable quantities of amino acids despite the dietary deficiency of tryptophan.

#### *Effect of tryptophan imbalance on growth of the mouse*

An amino acid imbalance was also produced in the mouse by the use of casein-oxidized casein diets. Results of these studies are presented in table 7. When mice were fed an 8% casein diet, growth of 8.4 gm was obtained in 4 weeks. The addition of 20% of oxidized casein to the diet, supple-

ing studies through successive generations. Matings were set up with one male and two female rats per cage. When pregnancy was recognized visually, by palpation, or from weight increments, the females were transferred to individual cages. If pregnancy was not established by the third week, the male was replaced. Following three (or occasionally 4) unproductive trials with females of known fertility, males were considered sterile and retired. Females were continued

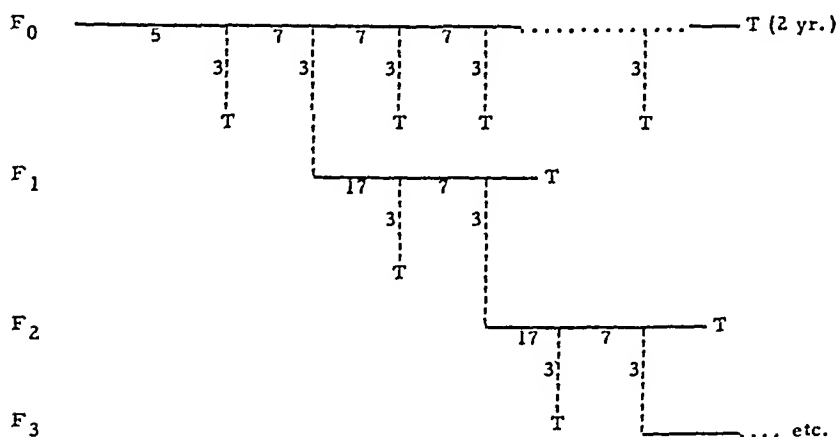


Fig. 1 Chronological scheme of reproduction and lactation. The horizontal lines represent the generations of rats through their successive matings and the dotted vertical lines indicate litters; termination of a litter or a generation is shown by the letter T; the figures indicate the number of weeks elapsed at each stage, beginning with the first mating in F<sub>0</sub>.

for a minimum of 6 matings with fertile males, even though some failures may have intervened.

Lactation was permitted for three weeks. Following weaning, death, or destruction of their litters, the females were allowed a one-week rest period before remating. In successive matings, the males were rotated among the females within their respective test groups.

As indicated in figure 1, matings continued in the F<sub>0</sub> generation throughout the entire two-year period. First litters were discarded at weaning. From the second litters of as many different mothers as possible, 10 rats of each sex were

ing action on methionine in the present experiments as was observed by the growth and corrective effects on the amino acid imbalance. When the methionine level in the diet was sufficiently reduced, an imbalance was produced even in the presence of vitamin B<sub>12</sub>. The addition of methionine to the diet corrected the imbalance.

Tryptophan imbalances are similar to methionine in this respect (Salmon, '54; Sauberlich and Salmon, '55). When the level of tryptophan in the diet was reduced sufficiently, an imbalance condition was produced regardless of the presence of niacin. Under these circumstances tryptophan, but not niacin, corrected the condition.

The imbalances of isoleucine and threonine were produced without any apparent unique vitamin-amino acid interrelationships. These results suggest that the imbalance effect may be a general phenomenon associated with specified conditions for probably most of the essential amino acids and possibly even for some of the "non-essential" amino acids. Results of the present study emphasize again that the amino acid requirements of the rat are not constant factors but are related to the diet employed and in particular to the protein or nitrogen level of the diet. Such an effect was demonstrated previously in this laboratory for the tryptophan requirement of the rat (Salmon, '54; Sauberlich and Salmon, '55). The mouse also appears to be subject to the effects of an imbalance in tryptophan. Although determinations of the quantitative increase in the requirements for methionine, isoleucine or threonine under the imbalance conditions were not made in the present investigation, such increased requirements are evident.

Several possible explanations were previously offered for the increased demand for tryptophan (Salmon, '54; Sauberlich and Salmon, '55). Such explanations are also applicable to the increased requirements noted for methionine, isoleucine and threonine. For example, a possible explanation was that in the absence of sufficient tryptophan to permit a balance of the ingested amino acids for synthesis into tissue proteins,



TABLE 1  
Summary of reproduction and lactation data of *F<sub>0</sub>* generation rats for six matings

EMULSIFIER OR FAT	NUMBER OF MATINGS	LITTERS BORN ALIVE	PUPS BORN ALIVE	AVERAGE NUMBER PUPS/LITTER		AVERAGE WEIGHT PUPS AT WEANING	FI <sup>1</sup>	GI <sup>2</sup>	VI <sup>3</sup>	LI <sup>4</sup>
				Born	Weaned					
None	119	76	654	8.6	6.5	gm 43.3	66	98	83	91
5% level										
Myrj 45	106	71	616	8.7	6.2	39.7	67	99	83	85
Myrj 52	114	89	875	9.8	6.4	39.7	79	99	71	91
Span 60	109	82	714	8.7	6.5	40.5	79	97	81	92
Tween 60	102 <sup>a</sup>	69	616	8.9	5.9	40.2	67	97	75	88
Tween 65	102	70	661	9.4	6.9	38.5	69	100	78	93
Tween 80	106	75	644	8.6	5.9	41.3	73	97	75	92
Mixture	88	71	624	8.8	6.5	41.8	82	99	80	93
Primex	116	83	761	9.2	5.9	44.1	72	99	73	89
10% level										
Myrj 45	112	83	765	9.2	5.7	40.3	75	99	66	91
Myrj 52	106	73	610	8.4	5.8	38.4	71	97	78	89
Span 60	112	84	735	8.8	5.3	37.5	79	94	64	94
Tween 60	111	79	752	9.5	5.6	38.0	79	100	67	88
Tween 65	109	70	684	9.8	5.1	40.0	65	99	60	88
Tween 80	112	68	617	9.1	6.6	41.0	63	97	75	96
Mixture	103	66	663	9.0	7.8	38.7	66	97	82	95
Primex	107	78	725	9.3	6.4	43.2	76	96	75	92
20% level										
Myrj 45	106	73	686	9.4	2.3	26.5	71	97	33	74
Myrj 52	118 <sup>a</sup>	61	534	8.8	4.3	37.0	53	98	55	90
Span 60	109	78	684	8.8	3.7	30.7	73	98	51	83
Tween 60	103	59	464	7.9	2.5	35.9	58	98	35	92
Tween 65	122 <sup>a</sup>	77	590	7.7	1.5	32.2	65	98	26	75
Tween 80	98	57	504	8.9	4.9	36.1	61	95	55	100
Mixture	103	66	591	9.0	3.4	32.9	64	100	45	84

<sup>1</sup> Fertility Index, (Pregnancies/Matings) 100.

<sup>2</sup> Gestation Index, (Litters born/Pregnancies) 100.

<sup>3</sup> Viability Index, (Pups surviving at 4 days/Pups born) 100.

<sup>4</sup> Lactation Index, (Pups weaned/Pups at 4 days) 100.

<sup>a</sup> These groups consisted of 21 females each; all other groups consisted of 20.

This was corrected by supplementation of the diet with the corresponding amino acids.

5. Plasma levels of methionine were not altered by the methionine imbalance. Tryptophan deficiency, however, caused a reduction in plasma amino acids.

6. The survival of adult rats fed protein-free diets was not influenced by supplements of certain amino acid-deficient proteins, glycine or urea.

7. An amino acid imbalance was produced in weanling mice fed a casein-oxidized casein diet. This imbalance could be largely prevented by supplements of tryptophan to the diet.

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Additional support for this conclusion may be seen in table 2. Here are shown the ranges in total number of litters cast throughout the two-year period by the females in the  $F_0$  generation, not limited, as in table 1, to the first 6 matings. Some produced as many as 9 litters whereas others, distributed rather uniformly among the test and control groups alike, proved to be sterile or nearly so. It may further be seen in table 2 that at least half of the females in each group (with two exceptions) gave birth to 4 or more litters. However, there was a tendency toward diminished productivity (in terms of numbers of litters) in groups receiving certain of the emulsifiers at the 20% level. The emulsifiers concerned, in increasing order of this effect, were Tween 60, Tween 80, and Myrj 52. It is pertinent to note that in these groups a distinct laxative response was seen. The feces of the affected rats were of varying degrees of softness, sometimes sufficiently fluid to be characterized as frankly diarrheal. Concomitantly, variable degrees of perianal inflammation and irritation developed.

Since the rats in the  $F_0$  generation were permitted to mate as long as they were productive, additional data on reproductive efficiency were obtained, namely the age at which loss of fertility occurred. This was estimated on the basis of two successive mating failures, specifically as of the day when the second mating was definitely established to be non-productive. While the precision of this estimate is far from exact, the data are nevertheless of interest. Table 3 shows that the  $F_0$  females were roughly  $450 \pm 50$  days old when their sterility was established. No significant differences were observed among the control groups and those receiving either 5 or 10% of emulsifier in their diets. While some tendency for a reduction in duration of fertility was noted with an increase in the emulsifier level to 20%, this was not marked, the average duration being in no case less than 376 days.

*Viability of the offspring.* Returning to table 1, it can be seen that the size of the litters, which ranged from 7.7 to

THE INFLUENCE OF  
AMINO ACIDS AND OTHER ORGANIC COMPOUNDS  
ON THE GASTROINTESTINAL ABSORPTION  
OF CALCIUM<sup>45</sup> AND STRONTIUM<sup>89</sup>  
IN THE RAT

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TWO FIGURES

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INTRODUCTION

Calcium utilization and bone mineralization are greatly reduced when the dietary protein is low in either quality or quantity (McCance et al., '42; Desikachar and Subrahmanyam, '49; Frandsen et al., '54). Poor calcium utilization also occurs on diets deficient in one or more of the essential amino acids (Bavetta et al., '54 and Haggard et al., '55). These amino acid insufficiencies result in a narrowing of the epiphyseal cartilage plate due to the reduction of cartilage cells, and a marked osteoporosis in both the epiphysis and diaphysis of the femur. Similarly, structural changes in the intestinal wall or a reduction of pertinent enzymes resulting from severe protein lack, or both, may interfere with mineral absorption.

Short term interactions between proteins or protein derivatives and calcium are indicated from the studies of Lehman and Pollack ('41-'42). These investigators observed that  $\alpha$ -amino acids increase the solubility of calcium salts and pro-

<sup>1</sup> On assignment from the United States Air Force Veterinary Corps.

<sup>2</sup> Under contract with the United States Atomic Energy Commission.

*Lactation.* At the 5% level of emulsifiers the L.I. values varied from 85 to 93%; at the 10% level the range was 88 to 96%, and at 20% it was 74 to 100%. Thus lactation efficiency, while quite high at all levels of emulsifiers, was moderately reduced in a few of the 20% groups, notably Myrj 45 and Tween 65. An indication of possible impairment of lactation at this feeding level may be seen in the somewhat lower weaning weights of the young. That neglect of the litters was a more significant factor than lactation failure *per se* is evident from the relatively greater drop in the V.I. values than in those for L.I. This was manifest to some extent even at the 10% emulsifier level. A much larger proportion of deaths among the newborn occurred shortly after birth, i.e. up to 4 days of age, than during the remainder of the 21-day nursing period. It would appear at least likely that the laxative effect with concomitant posterior ventral irritation at the high dosage level of some of the surfactants may also have had an adverse influence on the interest of the dams in caring for their offspring.

*Reproduction and lactation in successive generations.* The effect of the emulsifier diets on reproduction and lactation were made in rats of three generations descended from the first or parent generation ( $F_0$ ). As previously described, groups of 10 males and 10 females selected from the second litters of each generation constituted the progenitors. The data for the  $F_1$  and  $F_2$  generations are summarized in tables 5 and 6, respectively. Since the breeding experiments after the  $F_0$  generation were terminated when the second litters were weaned, the figures shown in these tables represent not more than two litters from each female, i.e. the product of 20 matings per group. For comparison, the responses to the first two matings in the  $F_0$  generation are shown in table 4; these observations are quite similar to those shown in table 1 for the first 6 matings in the initial generation, except for fertility which, as might be expected, tended to diminish as the rats grew older. In fact, comparison of tables 1 and 4 demonstrates that much of the relevant information on

with hydrochloric acid and all test solutions in each series were adjusted to equivalent acidities. Other reagents were C. P. grade.

## RESULTS

Tables 1 and 2 show the effects of essential and certain non-essential amino acids on the accumulation of  $\text{Ca}^{45}$  and  $\text{Sr}^{89}$  in rat femurs following simultaneous ingestion. Since the percentage increase was similar for both  $\text{Ca}^{45}$  and  $\text{Sr}^{89}$ , a

TABLE 1

*Effect of essential amino acids on the accumulation of  $\text{Ca}^{45}$  and  $\text{Sr}^{89}$  in rat femurs<sup>1</sup>*

TREATMENT	$\text{Ca}^{45}$	$\text{Sr}^{89}$	AVERAGE INCREASE IN $\text{Ca}^{45}$ AND $\text{Sr}^{89}$	$\text{Ca}^{45}/\text{Sr}^{89}$ IN FEMUR
	(% of dose in femur)	(% of dose in femur)	(% of control)	
Control	$4.6 \pm 0.2$	$2.8 \pm 0.1$	100	1.63
L-Lysine	$8.0 \pm 0.4$	$5.4 \pm 0.4$	182	1.48
L-Arginine	$7.4 \pm 0.3$	$5.5 \pm 0.4$	176	1.33
L-Tryptophan	$7.3 \pm 0.4$	$4.5 \pm 0.4$	159	1.62
L-Leucine	$6.6 \pm 0.2$	$4.0 \pm 0.3$	142	1.62
L-Histidine	$6.3 \pm 0.2$	$3.3 \pm 0.1$	126	1.89
L-Methionine	$6.0 \pm 0.3$	$3.1 \pm 0.2$	119	1.96
L-Isoleucine	$5.3 \pm 0.2$	$3.1 \pm 0.3$	112	1.70
L-Valine	$5.4 \pm 0.2$	$2.9 \pm 0.1$	110	1.84
L-Threonine	$5.1 \pm 0.2$	$2.9 \pm 0.2$	107	1.78
L-Phenylalanine	$5.3 \pm 0.2$	$2.8 \pm 0.2$	105	1.91

<sup>1</sup> Values represent mean  $\pm$  standard error of the mean; 8 animals per group; mean body wt. =  $92 \pm 1$  gm; mean femur ash wt. =  $184 \pm 4$  mg; dose contained 10 mg carrier  $\text{CaCl}_2$  and 0.84 millimoles of amino acid.

single average value is given for brevity. It will be noted that lysine and arginine were most effective in promoting the appearance of the radioisotopes in the bone; these two amino acids almost doubled the  $\text{Ca}^{45}$  and  $\text{Sr}^{89}$  values in the femur. Tryptophan, leucine, and aspartic acid were also appreciably effective. The other amino acids studied had lesser or no significant effect. It should be noted that the administration of lysine resulted in an occasional diarrhea; this was not observed with any other amino acid.

TABLE 5  
Summary of reproduction and lactation data of *F<sub>1</sub>* generation rats for two matings

EMULSIFIER OR FAT	NUMBER OF MATINGS	LITTERS BORN ALIVE	PUPS		NUMBER PUPS PER LITTER		AVERAGE WEIGHT PUPS AT WEANING	F.L. <sup>1</sup>	G.L. <sup>1</sup>	V.L. <sup>1</sup>	L.L. <sup>1</sup>
			Born alive	Weaned	Born	Weaned					
None	20	18	182	107	10.1	6.0	gm 40.9	90	100	86	69
5% level											
Myrj 45	20	19	196	125	10.3	6.6	38.4	95	100	82	78
Myrj 52	20	18	164	95	9.1	5.3	37.2	90	100	79	73
Span 60	20	19	199	139	10.5	7.3	37.8	95	100	79	89
Tween 60	19	15	154	92	10.3	6.1	36.3	85	92	69	86
Tween 65	20	18	159	113	8.8	6.3	42.7	90	100	84	81
Tween 80	20	15	149	126	10.0	8.4	37.7	75	100	93	91
Mixture	19	18	174	116	9.7	6.5	36.8	95	100	78	86
Primex	20	15	131	92	8.8	6.1	44.8	80	94	70	100
10% level											
Myrj 45	20	20	205	99	10.3	5.0	37.9	100	100	64	76
Myrj 52	20	19	222	100	11.7	5.3	36.4	95	100	73	62
Span 60	20	18	188	127	10.4	7.1	31.8	100	90	82	82
Tween 60	20	18	200	101	11.1	5.6	36.2	90	100	61	83
Tween 65	20	17	185	100	10.8	5.9	37.7	85	100	59	92
Tween 80	18	18	191	144	10.6	8.0	35.2	90	100	97	87
Mixture	20	14	142	75	10.1	5.4	38.9	70	100	68	78
Primex	20	15	161	116	10.7	7.7	36.7	80	93	80	90
20% level											
Myrj 45	22	13	142	28	10.9	2.2	28.1	59	100	28	70
Myrj 52	20	14	131	83	9.3	5.9	33.5	75	93	68	93
Span 60	20	17	145	75	8.5	4.4	31.5	85	100	60	86
Tween 60	20	12	103	36	8.5	3.0	37.4	60	100	41	86
Tween 65	20	13	147	31	11.3	2.4	33.1	75	87	35	60
Tween 80	20	12	133	87	11.0	7.2	32.3	60	100	71	92
Mixture	20	13	130	22	10.0	1.7	30.5	65	100	28	59

<sup>1</sup> See footnotes, table 4.

Table 3 summarizes a comparison of lysine, arginine and leucine with other organic compounds that have been reported to influence calcium absorption. It may first be noted that the results with the amino acids showed good agreement with other experiments (tables 1 and 2). Of particular interest was the action of lactose, which was more effective than either lysine or arginine, and which increased the radioisotope content of the femur by a factor of about 2.5. The lactose effect on calcium absorption and utilization has been generally accepted and recently emphasized by Fournier ('55).

TABLE 4

*Effect of lysine on femur accumulation of oral versus parenterally administered  $\text{Ca}^{45}$  and  $\text{Sr}^{90}$* <sup>1</sup>

TREATMENT	METHOD OF $\text{Ca}^{45}$ AND $\text{Sr}^{90}$ ADMINISTRATION	$\text{Ca}^{45}$	$\text{Sr}^{90}$	AVERAGE INCREASE IN $\text{Ca}^{45}$ AND $\text{Sr}^{90}$	$\text{Ca}^{45}/\text{Sr}^{90}$ IN FEMUR
		(% of dose in femur)	(% of dose in femur)	(% of control)	
Control	Oral	$4.4 \pm 0.4$	$2.5 \pm 0.3$	100	1.78
L-Lysine	Oral	$8.0 \pm 0.2$	$5.4 \pm 0.1$	199	1.47
Control	I. P.	$8.2 \pm 0.3$	$7.5 \pm 0.3$	100	1.10
L-Lysine	I. P.	$9.0 \pm 0.3$	$7.7 \pm 0.1$	107	1.17

<sup>1</sup> Values represent mean  $\pm$  standard error of the mean; 6 animals per group; mean body wt. =  $138 \pm 4$  gm; mean femur ash wt. =  $229 \pm 14$  mg; dose contained 10 mg  $\text{CaCl}_2$  and 0.84 millimoles of amino acid.

The sodium lactate and sodium gluconate showed only small positive effects; the vitamin B mixture and sodium citrate had no effect. The negative results with citrate tend to corroborate the findings of Antoni and Cremer ('55).

The appearance of ingested  $\text{Ca}^{45}$  and  $\text{Sr}^{90}$  in the bone can be theoretically related to absorption from the gut, and also to any other processes concerned with the removal of the radioisotopes from the blood; for example, exchange into extravascular spaces and excretion. When animals to be compared are under similar physiological conditions, it is generally accepted that the appearance of ingested  $\text{Ca}^{45}$  and  $\text{Sr}^{90}$  in bone is a reliable index of absorption of these radio-



reproduction and lactation can be obtained from only the first two litters in a generation.

Comparison of the reproduction data in tables 4, 5, and 6 shows that the responses in the three successive generations were quite similar. The proportion of matings resulting in pregnancy tended to be lower at the 20% emulsifier level although this effect was less noticeable in the case of Myrj 45 and Span 60 than in the other emulsifier groups. That the third generation was generally less productive than the first two may be seen in the lower F.I. values for the Primex as well as the emulsifier groups.

Nearly, if not exactly, 100% of pregnancies went to term in all generations. The trend toward higher mortality during the 4 days post partum as the level of emulsifier increased, was not as marked in the  $F_1$  and  $F_2$  as in the  $F_0$  generation. The proportion of nurslings surviving the lactation period was reduced in some cases in the  $F_2$  generation at the 20% emulsifier level (e.g. Myrj 45, Span 60, Tween 65).

Compared with the other 20% emulsifier groups, the one receiving Tween 80 evidenced a striking superiority in respect to survival of young from birth to weaning age; whereas the Myrj 45, Tween 65 and mixed emulsifier groups appeared to respond most poorly.

Reproductive performance in general appeared to be inferior in the third generation rats compared to their progenitors, in both the emulsifier and Primex series.

The reproductive performance of the  $F_3$  generation was not investigated because the rats in this generation were sacrificed either at weaning or at the end of the 12-week growth period.

*The effect of increasing the fat level of the basal diet on post-partum survival.* In order to determine whether the effects observed at the 20% emulsifier levels, especially in relation to fertility and viability, might have been due to the low (4.0%) fat content of the basal, unsupplemented diet, further tests were conducted on the  $F_2$  generation rats after their second litters were weaned. These groups of about 10

however, that the calcium and strontium ions do not respond equally to the processes causing the increased absorption. This may be related to ionic characteristics, the presence of the carrier calcium, or discriminating processes in the absorption mechanism.

Figure 2 presents a dose-response curve for lysine and the  $\text{Ca}^{45}$  and  $\text{Sr}^{80}$ . The conditions of this experiment were the same as those previously described, with the exception that

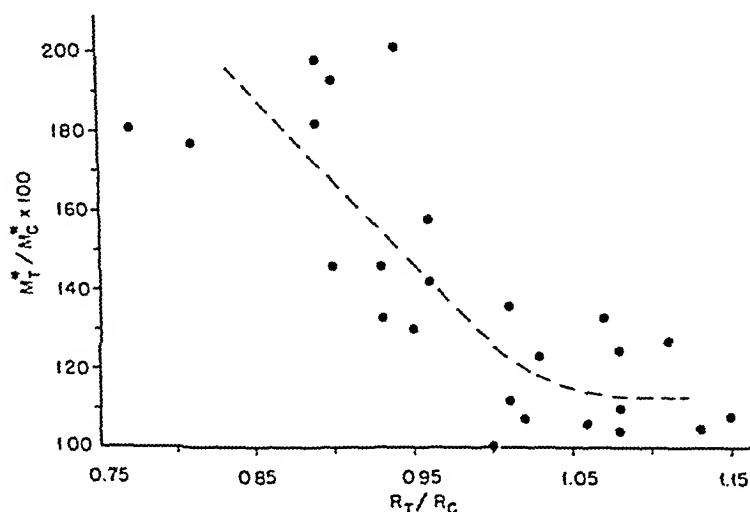


Fig. 1 Scattergram of the correlation between the  $\text{Ca}^*:\text{Sr}^*$  ratio and percentage increase in accumulated  $\text{Ca}^{45}$  and  $\text{Sr}^{80}$  in rat femurs.  $\text{Mt}^*$  = average percentage dose of  $\text{Ca}^{45}$  and  $\text{Sr}^{80}$  in bones of treated group and  $\text{Mc}^*$  = average percentage dose of  $\text{Ca}^{45}$  and  $\text{Sr}^{80}$  in control group.  $R_t/R_c$  =  $\text{Ca}^*:\text{Sr}^*$  ratio in treated group divided by  $\text{Ca}^*:\text{Sr}^*$  ratio in control.

the carrier  $\text{CaCl}_2$  was increased to 60.8 mg to reduce the effect of endogenous calcium. The varying amounts of L-lysine and the molar ratios of L-lysine to  $\text{CaCl}_2$  are shown in the graph. There appeared to be little effect at a lysine to calcium molar ratio of less than one half; the greatest increase in absorption occurred as the ratio increased from 1 to 2. This suggests that the action of lysine is other than that due to a vitamin-like stimulation; one would expect a much lower effective lysine to calcium ratio if this were true.

males and 10 females were divided into two subgroups. One was allowed to continue on the same diet, whereas the other received an addition of 9% of Primex (the level computed as isocaloric with 20% Myrj 45), in replacement of an equal proportion of the wheat and corn component of the basal diet. These subgroups were then mated as before to produce two additional litters from each female.

Table 7 shows the number of matings actually set up in each subgroup and the results of these matings. There was no consistent relation between the responses of the 20% emulsifier groups to the third and 4th matings as compared to the first two (table 6). The F.I. values rose in two cases (Span 60 and Tween 60), fell in three groups (Myrj 45, Tween 80 and the Mixture), and were practically unchanged in the other two groups (Myrj 52 and Tween 65). The V.I. values dropped in three instances (Myrj 52, Span 60 and Tween 80) and showed little or no change in the remaining groups. However improvement in lactation was observed in all groups with the exception of the Tween 65 group which fell off somewhat and the Tween 80 group which, quite surprisingly in view of its previously high L.I. values, showed complete failure.

As regards the influence of the addition of dietary fat on the reproductive responses, it is apparent from the F.I. values in table 7 that little if any increase on the proportion of successful matings was observed. Post partum survival was improved in 4 of the emulsifier groups (Myrj 45, Span 60, Tween 60, and Tween 80) as a result of the addition of fat, while the remainder showed little change. No striking effects on the L.I. values were observed from the addition of fat to the 20% emulsifier diets with the exception of the Myrj 52 and Tween 65 groups where decreases were noted.

In considering these somewhat erratic responses to fat supplementation in the third and 4th matings it is necessary to take into account the fact that the subgroups were of smaller size than the original groups. In any event it can be stated that the most common finding appeared to be improved via-

provided by the amino acid is primarily responsible for the increased mineral absorption.

Examination of the metabolic and physicochemical characteristics of these compounds as related to their effect on mineral absorption permits some limited interpretations concerning the mechanisms. The theory of Lehmann and Pollack ('41-'42), relating increased mineral absorption to the increased solubility of the calcium salts in the presence of  $\alpha$ -amino acids, cannot entirely explain the present data. Glycine, which has a pronounced solvent action on calcium salts, was relatively ineffective in these studies. Except for the dicarboxylic amino acids, the other amino acids, especially the basic types, would probably be no more effective than glycine in the solution of calcium salts. Complex formation between the amino acid *per se* and the mineral also cannot alone account for the present observations. This is based on inferences from the data of Li and Doody ('52) in which it was shown that lysine and arginine form unstable complexes with the cupric ion, whereas glutamic acid forms a stable complex. Preliminary observations in this laboratory indicate that calcium and strontium are similar to copper in this respect. Since lysine and arginine (isoelectric points of 9.74 and 10.76, respectively) are in the cationic form under the pH conditions of the intestine, strong complex formation between these basic amino acids and calcium would be theoretically unexpected. As pointed out by Greenberg ('44), in the main, electrostatic forces can be considered as acting to prevent or retard the ionization of alkaline earth cations. From these considerations, it appears that the effectiveness of aspartic acid and glutamic acid may be related to complex formation, but one must look elsewhere to understand the mechanism of the stimulation by lysine and arginine.

The passage of the amino acid itself through the gut barrier is quite likely associated with its effect on mineral absorption. It has been shown with everted gut sacs *in vitro* that glycine and the L-isomers of alanine, phenylalanine, methionine, histidine, isoleucine, and proline are "actively" transported

were noted, however. For example, the relatively low L.I. values for the basal control group in the  $F_1$  generation, for the 10% Primex group in the  $F_2$  generation, and for the 5 and 20% (but not the 10%) Myrj 45 groups in the  $F_2$  generation. These scattered observations were not limited to the emulsifier groups nor were they graded to dosage level. They may therefore be regarded as falling within the range of normal biological variation.

#### SUMMARY AND CONCLUSIONS

Breeding studies were undertaken in successive generations of rats on diets containing partial ester emulsifiers (Myrj 45 and 52, Span 60, and Tween 60, 65 and 80) to determine whether their chronic ingestion at levels up to 20% might induce cumulative or subtle effects manifested only under the conditions of physiological stress thus imposed. The responses were assessed, *inter alia*, in terms of indexes representing the proportions of matings resulting in pregnancy (fertility), pregnancies resulting in live litters (gestation), young remaining alive at 4 days (viability), nurslings weaned in relation to the number alive at 4 days and their weights at weaning (lactation).

On the average, 7 out of 10 matings were successful in both control and emulsifier groups, regardless of the level of dietary supplementation. Practically all pregnant rats cast live litters. The reproduction and lactation responses in all emulsifier groups at the 5% level were no different from those of the controls. Probably because of maternal neglect, survival of newborn litters was somewhat diminished in several of the emulsifier groups at the 10% level (Myrj 45, Span 60, and Tween 65) and in all of them at 20%. At the highest level some impairment in lactation efficiency was evidenced in most groups by the lower weaning weights; and in the Myrj 45, Tween 65, and mixed emulsifier groups also by greater mortality of the nurslings.

Similar responses with respect to survival and lactation were noted in the two succeeding generations. Despite the

The effect of lactose on calcium absorption and utilization has not been definitively explained. Fournier ('55) suggested that lactose, galactase, and certain pentoses increase calcium utilization by a favorable metabolic effect on ossification; however, the foregoing studies were of longer duration than those reported in this paper and may not be entirely applicable. More classical explanations of lactose action involve the formation of a more acid condition in the gut, which in turn promotes calcium absorption (Maynard, '51). Gluconate and lactate probably increased the absorption of  $\text{Ca}^{45}$  and  $\text{Sr}^{89}$  by virtue of the greater solubility of the salts; little is known of the transport mechanism for either lactate or gluconate. Citrate, which forms a soluble complex with calcium, did not increase  $\text{Ca}^{45}$  or  $\text{Sr}^{89}$  absorption. This would suggest that complex formation is not necessarily a decisive factor in promoting mineral absorption. Other factors, such as the movement of the complex itself, must be considered. The lack of effect from the administration of vitamin B was expected since the test animals were not depleted of these nutrients under the present experimental conditions.

Although the practical implications of these results are not clear at this time, the data certainly offer an additional explanation for the favorable effect of protein or protein derivatives on calcium metabolism. Further studies are in progress to elucidate the mechanism of action, and to observe interrelationships with other factors, such as vitamin D and phosphorus.

#### SUMMARY

1. Eighteen amino acids, including those essential for the rat, were assayed for effect on the gastrointestinal absorption of  $\text{Ca}^{45}$  and  $\text{Sr}^{89}$ . The minerals and amino acid were ingested simultaneously; radioassay values for the femur obtained 24 hours after dosage were used as a measure of absorption.

2. L-Lysine and L-arginine were the most potent in promoting mineral absorption, approximately doubling the  $\text{Ca}^{45}$  and



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cernible destruction of amino acids. Consequently, several procedures were developed to take into account the enzymatic availability of component amino acids (Dunn and Rockland, '47; Anderson and Williams, '51; Horn et al., '52; and Halevy and Grossowicz, '53). The results obtained with these methods generally did not correlate well with the biological value of proteins as determined by rat assay. The values reported by Horn et al. ('52) were in good agreement with the relative protein efficiency of heat processed cotton seed meals; however, no evidence was presented concerning the general applicability of the method.

A procedure for the *in vitro* estimation of the net utilization of proteins was reported by Sheffner et al. ('55, '56). This method referred to as the Pepsin Digest-Residue (PDR) index was derived by integration of the pattern of amino acids released by *in vitro* pepsin digestion with the amino acid pattern of the remainder of the protein. The new index gave excellent correlation with the net utilization value of the proteins studied. The present study demonstrates that the PDR index also measures changes in net protein utilization which occur during heat processing and storage.

#### METHODS AND MATERIALS

Acid and alkaline hydrolysates and enzyme digests were prepared as previously described (Sheffner et al., '56), except that pancreatin (USP) was used where trypsin was formerly indicated; also, alkaline hydrolysis for tryptophan and tyrosine was extended to 8 hours at 120°C. with 5 N NaOH. Nitrogen was measured by a macro-Kjeldahl procedure in which mercuric oxide was used as the digestion catalyst. Individual amino acid analyses were performed by the microbiological procedures of Sheffner et al. ('48) as subsequently modified ('56).

The test protein materials used in this study were: vitamin-free casein,<sup>4</sup> low-temperature solvent-extracted soybean meal

<sup>4</sup>Labco brand, The Borden Company.

# DIGESTIBLE ENERGY IN RELATION TO FOOD INTAKE AND NITROGEN RETENTION IN THE WEANLING RAT<sup>1,2</sup>

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The energy content of a ration appears to exert a considerable influence upon both food consumption and protein utilization. Several experiments with the chick have indicated that the productive energy content of the ration is a major factor in controlling feed intake. Hill and Dansky ('50, '54), and Dansky and Hill ('51), have pointed out the remarkable ability of the chick to compensate for reduced dietary energy level by increasing feed consumption. Similarly, Peterson et al. ('54) noted that feed intake increased to satisfy the energy needs of chicks, though when the rations were of a very low energy content the birds were unable to consume sufficient feed to satisfy their energy requirements.

Investigations relating to the effects of energy levels on food consumption, in species other than the chick, have not been widely developed. Hegsted and Haffenreffer ('49), working with rats, stated that, "the food intake of an animal is governed by means yet unknown at a certain percentage above its normal basal metabolism." In the same paper it is suggested that, "the mean daily calorie intake varied as the mean body weight raised to the 0.88 power." Cowgill ('28)

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terminated by the Mitchell ('24) method of nitrogen balance in rats were similarly lowered. The PDR index of casein was not appreciably changed when the casein was autoclaved at 250°F. for 30 minutes or for 20 hours. Net utilization values were not obtained for these samples; however, Chick et al. ('35) reported little, if any, change in the biological value or digestibility of casein heated at 250°F. for as long as 72 hours.

The effect of processing and storage upon beef and a beef with spaghetti mixture is presented in table 2. For fresh raw beef, a PDR index value of 76 was obtained. This checks closely with the net utilization values obtained by Mitchell

TABLE 1

*The effect of heat treatment upon the PDR index and net utilization of casein*

TREATMENT	PDR INDEX	NET UTILIZATION
None	68	82
Oven, 350°F., 40 min.	60	
Oven, 350°F., 1 hr.	39	44
Oven, 350°F., 5 hrs.	23	24
Autoclave, 250°F., 30 min.	71	
Autoclave, 250°F., 20 hrs.	66	

and co-workers ('49) and Mayfield and Hedrick ('49). Pan-fried beef was not significantly different from the control. In this respect, both Mitchell et al. and Mayfield and Hedrick have reported that roasting does not reduce the net utilization of beef protein. In the case of the mixed beef and spaghetti, there was a decrease in the PDR index from 72 to 66 following processing and a further decrease to 60 after storage for 6 months at 118°F. These changes in PDR index reflected the drop in net utilization as measured by rat assay.

The PDR index and net utilization value of raw and heated soybean meals are presented in table 3. The PDR index of soybean meal steamed for 30 minutes was the same as the net utilization value. Soybean meal autoclaved for 8 hours showed an equivalent decrease in both the PDR index and the net

Feces were collected daily, dried in an air oven at 105°C. and then ground to a fine powder.

TABLE 1

*Rations fed during acclimatization and metabolism periods*

	RATION 1	RATION 2
Nitrogen source "A", <sup>1</sup> %	15.2	15.2
Sucrose, %	54.8	44.8
Alphacel, <sup>2</sup> %	20.0	30.0
Mazola oil, %	5.0	5.0
Salts, <sup>3</sup> %	4.0	4.0
Vitamin mix, <sup>4</sup> %	1.0	1.0
Analysis		
Gross energy, Cal./gm	4.22	4.23
Nitrogen content, gm %	2.07	2.08

<sup>1</sup> Nitrogen source "A." Casein 58.7 gm (~8 gm N), lactalbumin 68.8 gm (~8 gm N), DL-methionine 0.3 gm, L-histidine HCl 1.5 gm, DL-threonine 1.0 gm. Calculated to supply the amino acid requirements of the rat when expressed as a ratio to lysine = 1.0 (Rose, '38; Block and Bolling, '51).

<sup>2</sup> Alphacel "non-nutritive cellulose." Nutritional Biochemical Corporation, Cleveland, Ohio.

<sup>3</sup> Jelinek et al., '52.

<sup>4</sup> Vitamin mix modification of Jelinek et al., '52. Vitamin B<sub>12</sub> at a level of 0.03 mg/kg of food replaced Wilson's whole liver powder.

## RESULTS AND DISCUSSION

The data which follow refer solely to the 7-day metabolism period. Table 2 lists the principal mean values obtained during this experiment. The initial weight of the rats was the weight at the start of the 7-day metabolism period. Digestible energy consumption was determined by subtracting the total fecal energy from the gross energy intake. Digestible nitrogen consumption was calculated in a similar manner to digestible energy, while nitrogen retained was equivalent to nitrogen digested minus total urinary nitrogen. It should be noted that the term "digestible" refers to apparent and not true digestibility.

Ration 1 with an Alphacel content of 20% had a total digestibility of 78% while ration 2, containing 30% of Alphacel, had a digestibility level of 68%. That an increase of 10%

PDR index, only the pepsin digest and total amino acid results are used, the question arose as to whether a correction for trypsin digestion should be introduced into the PDR index to account for the effects of anti-tryptic factors in raw soybean meal.

In an attempt to answer this question, the soybean samples were treated with pepsin as usual, then adjusted to pH 8.2 and incubated with pancreatin for 24 hours at 37°C. The

TABLE 4  
*Effect of optimal heating upon the enzymatic release of amino acids from soybean meal*

AMINO ACID	COMPLETE HYDROLYSIS		PEPSIN		PEPSIN PLUS PANCREATIN	
	Raw	Steamed <sup>1</sup>	Raw	Steamed	Raw	Steamed
	mg/gm	mg/gm	per cent liberation		per cent liberation	
Cystine	12.8	12.4	2.3	1.6	4.7	21.0
Lysine	59.1	59.5	2.0	1.7	20.6	68.9
Histidine	29.2	30.8	2.4	2.0	17.1	33.8
Valine	57.8	56.5	16.3	15.4	36.7	56.8
Methionine	13.2	13.5	15.9	14.1	36.4	51.1
Isoleucine	54.0	54.5	47.6	47.5	68.2	89.9
Leucine	77.3	78.0	57.6	60.3	77.8	96.4
Tyrosine	31.0	30.9	13.9	13.9	66.8	81.6
Tryptophan	16.8	17.0	22.6	22.4	43.4	51.2
Phenylalanine	57.0	59.7	17.7	16.8	44.9	50.2
Threonine	37.9	39.0	53.8	48.7	74.9	84.1

<sup>1</sup> Steamed at atmospheric pressure (212°F.).

total amino acid composition of the proteins and the percentage liberation of amino acids from the raw and steamed soybean meals by the pepsin and the pepsin plus pancreatin treatments are presented in table 4. Whereas there is no change in the quantity of amino acids in the completely hydrolyzed protein nor in the amount of amino acids released by pepsin, there is a considerable increase in the amount of amino acids released from the steamed soybean meal by the pepsin plus pancreatin treatment. The results also show that this increased liberation following pancreatin treatment varies with the individual amino acids, and in this respect

The results of the analysis of covariance indicate that the food intake of weanling rats, receiving rations containing approximately 2.07% nitrogen and varying in Alphacel content by 10% (20% and 30%), was significantly influenced by the digestible energy content of the food; that is, it would appear that the rats ate to satisfy their energy requirement. It is anticipated that the increase in food consumption, resulting from a reduction in the digestible energy content of the ration, could only occur within certain physiological limits; this having already been demonstrated for the chick (Peterson et al., '54).

TABLE 3

*Analyses of variance and covariance of food consumption and digestible energy consumption between two rations differing in Alphacel content (non-nutritive cellulose)*

ANALYSES	SOURCE OF VARIATION	NON-ADJUSTED		ADJUSTED FOR INITIAL WEIGHT	
		Degrees of freedom	Mean square	Degrees of freedom	Mean square
Food consumed	Between rations	1	12	1	263 <sup>1</sup>
	Within rations	13	79.4	12	43.6
Digestible energy consumed	Between rations	1	2307	1	124
	Within rations	13	700	12	284

<sup>1</sup> Significant at 5% level.

It was also desired to determine the influence which the digestible energy consumption may have had on the amount of nitrogen retained. The correlation between these factors was 0.928 for ration 1 (20% Alphacel) and 0.910 for ration 2 (30% Alphacel). The pooled correlation for both rations was 0.918. All these correlations were very large and highly significant indicating a strong association between digestible energy consumption and retained nitrogen.

Since initial weight might have influenced both digestible energy consumption ( $r = 0.829$ ) and nitrogen retention ( $r = 0.720$ ), it was necessary to remove the effects of initial weight from the correlation between digestible energy consumption and nitrogen retention. This was accomplished by calculating

anti-tryptic factors of raw soybean meal introduce differences in the rate of release of individual amino acids, these differences do not significantly influence the biological value of the raw protein. On the basis of the results reported here, a correction for tryptic digestion would not be expected to improve the accuracy of the PDR index for predicting the net utilization value of raw soybean meal.

Overestimation by the PDR index of the net utilization value of raw soybean meal is probably best explained as being due to the presence in the meal of a toxic factor or factors. Such toxic factors have been experimentally demonstrated and shown to be sensitive to heat. (Liener et al., '49; Liener, '53; Desikachar and De, '47; Klose et al., '48; Borchers et al., '48; Westfall et al., '48). Consequently, the PDR index should also be an accurate indicator of the net protein utilization in soybean preparations in which the toxic factor has been destroyed by heat treatment.

The particular advantage of the nitrogen balance method for measuring biological value (Thomas, '09; Mitchell, '24) over other biological assay methods is that it determines directly the storage of protein in growth rather than assuming that this storage is proportional to body weight gains. The procedure also distinguishes between loss of nitrogen in the digestive process, i.e., undigested plus secretory protein, and losses due to the remaining metabolic processes of the animal body. However, for purposes of appraising the value of a food as a source of dietary protein, a single figure for the net protein utilization has distinct advantages (Mitchell, '44). For most food proteins the distinction between biological value and net utilization is academic since their coefficients of digestibility are very high. However, in the case of heat-processed foods in which protein digestibilities are significantly reduced it is important for practical nutritional considerations to measure the net utilization rather than the biological value. The PDR index which measures the net utilization of proteins directly is a useful procedure for estimation of the nutritional quality of both natural and processed proteins.

## SUMMARY

It has been demonstrated that the food intake of two groups of weanling rats, whose rations contained respectively 20 and 30% of non-nutritive cellulose, was significantly influenced by the digestible energy content of the food. This would indicate that within physiological limits, as yet not determined, weanling rats eat to satisfy their energy requirements.

Digestible energy consumption has been shown to influence the nitrogen retention of the weanling rat. Approximately 69% of the variation in the nitrogen retention of the weanling rats used in this experiment was associated with digestible energy consumption when the effects of initial weight were removed.

It is postulated that within limits there is an optimum digestible energy level for each nitrogen level of a ration when the criterion of measurement is nitrogen retention.

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STUDIES IN CALCIUM METABOLISM.  
EFFECT OF FOOD PHYTATES ON CALCIUM<sup>4,5</sup>  
UPTAKE IN BOYS ON A MODERATE  
CALCIUM BREAKFAST<sup>1,2,3,4</sup>

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TWO FIGURES

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In an earlier study (Bronner et al., '54) it was reported that phytates significantly depressed the calcium uptake when the test breakfast contained approximately 85 mg of calcium (Ca) and 100 mg of phytic phosphorus. We are now reporting on

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<sup>3</sup>The data in this publication are taken from the dissertation presented (1952) by Felix Bronner to the Department of Food Technology, Massachusetts Institute of Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>4</sup>Authorization for the use of restricted quantities of Ca<sup>45</sup> in patients institutionalized for mental inadequacy was granted through the Subcommittee on Human Applications by the Isotope Division of the Atomic Energy Commission. The Ca<sup>45</sup> was obtained on allocation from the Oak Ridge National Laboratory.

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fat, carbohydrate, protein or cholesterol intake and the serum cholesterol level.

In the studies relating age and serum cholesterol most workers agree that in women, at least, the serum cholesterol level rises with age. Sperry and Webb ('50), who studied the serum cholesterol levels of 14 men and 8 women in 1934-'36 and again in 1949, reported that in 6 of the men and in 6 of the women increases of 15 to 30% were found in the second survey. In the work of Gram and Leverton ('49) and of Garcia et al. ('55), with women on a self-selected diet, a significant rise in serum cholesterol with age was shown. Hobson et al. ('53) reported that the mean serum cholesterol levels of their subjects (elderly men and women living at home) were significantly higher than those of control groups of younger people.

Work with animals has suggested a relationship between cholesterol and ascorbic acid in the body metabolism. Booker et al. ('51) found an increase in serum cholesterol in both rats and dogs after administration of ascorbic acid, and Consuelo Mendoza ('52) demonstrated that injection of rabbits with ascorbic acid also resulted in a rise in serum cholesterol. Gillum et al. ('55), in their study of older women, reported a slight positive relationship between serum cholesterol and serum ascorbic acid.

In the present study a comparison was made of the serum cholesterol levels of women of two different age groups offered the same institution diet. The effect of supplementation of the diet with various levels of ascorbic acid on the serum cholesterol in the two groups was also studied.

#### PROCEDURE

The subjects for this study were 29 women in a state training school for the handicapped<sup>3</sup>; all were in good physical health. They were divided into two groups according to age. In the younger group of 15 subjects the average age was 31 years, with a range of 28 to 34. In the older group of 14 subjects the average age was 64 years, with a range of 56 to 77.

averaged 7.6 years, with a range of 5.0 to 9.8 years. If the 8th boy is included (subject 33) the respective group averages were 12.8 years, 40.2 kg and 7.1 years.

The subjects had been prepared for the study by receiving a daily supplement of one multivitamin tablet<sup>6</sup> and of one quart of milk, for a period extending from two weeks preceding the study period to the end of the experiment. Analysis of the diet served at the school revealed that it very nearly met the Recommended Daily Dietary Allowance of the National Research Council ('48) for children of this age group.

### *Experimental meals*

Two breakfasts were given: an oatmeal breakfast (O) with phytate naturally present in the cereal, and a farina breakfast (F) which contained no phytate. Table 1 shows the composition of the breakfasts.

The radiocalcium ( $\text{Ca}^{45}\text{Cl}_2$ ) was added to approximately 60 ml of the milk which was then mixed intimately with the cereal. The children drank the remainder of the milk as they ate the cereal.

### *Sample collection*

*Blood* was drawn by venipuncture at about 2.5 hours postprandially. It was allowed to clot and, following centrifugation, the serum was analyzed for its content of Ca and  $\text{Ca}^{45}$ .

*Urine* was collected daily for 5 days. Ca and  $\text{Ca}^{45}$  analyses were carried out on the pooled samples collected during the first three days, and also on pooled samples of days 4 and 5<sup>7</sup>.

*Feces* were collected daily for 5 days and a pooled 5-day specimen was analyzed for its content of Ca and  $\text{Ca}^{45}$ .

Samples were preserved and handled in the manner described previously (Bronner et al., '54). The quantities of  $\text{Ca}^{45}$  given in these studies were so low that the level of

<sup>6</sup> Vi-Penta Perles Forte, generously donated by Hoffman-LaRoche, Inc.

<sup>7</sup> Because of the very low radioactivity of the urine samples collected on days 4 and 5, the analytical results are not reported, nor were they included in the statistical evaluation.

lated for the diets, using the U.S.D.A. Agriculture Handbook No. 8 (Watt and Merrill, '50). The cholesterol content of the diets was calculated from the tables of Okey ('45) and from data provided by Gillum ('55).<sup>4</sup> Analysis of food samples collected in the dining room from time to time throughout the experimental period yielded information for calculating the vitamin C content of the diets. The method used in these determinations was the 2,4-dinitrophenylhydrazine method of Roe and Kuether ('43), using *norit* oxidation.

Venous blood samples for serum cholesterol determinations were taken after 7 weeks on the 32-mg level of ascorbic acid intake, and following the 25-, 50-, and 75-mg supplement levels. The first three samples were taken at intervals of one month. The 4th sample was taken two weeks after the third. The samples were always taken at 10:00 A.M., three hours after breakfast. A modification of the method of Kibrick, Roberts and Skupp ('51)<sup>5</sup> was used to determine the total serum cholesterol in duplicate 150-mm<sup>3</sup> samples of serum. The samples were read in a Coleman Jr. spectrophotometer. Serum ascorbic acid was determined according to the method outlined in the Northeast Regional Publication on Techniques ('51).

Height and skeletal build were recorded during a physical examination of the women made at the beginning of the study. Each subject was weighed every month. The percentage deviation from the desirable weight was calculated for each subject by means of the Metropolitan Life Insurance Tables (Metropolitan Life Insurance Co., '42).

## RESULTS

The women in the older group had a higher serum cholesterol level than the younger women throughout the study. The mean serum cholesterol level for the older women was

<sup>4</sup>Cholesterol values for foods provided by Dr. Helen L. Gillum were from data compiled by the California Agricultural Experiment Station for use in the Western Regional Research Project W4.

<sup>5</sup>Dr. Mary M. Clayton of the Maine Agricultural Experiment Station assisted in the modification of this method.

radioactivity of the urine and stool specimens approached background in about 5 days and collections were therefore discontinued.

### *Analytical procedures*

The analytical techniques have already been described (Bronner et al., '54). All counting data are reported as corrected to the time of ingestion of  $\text{Ca}^{45}$ . Decay corrections were made with the aid of suitable  $\text{Ca}^{45}$  standards used in all counting runs.

In the radiochemical determinations, experimental difficulties caused poor precision in some serum and urine samples. Serum samples in both the experiments, but particularly in experiment A, tended to gel during the preparative stage. Limitation in sample size often made repeat determinations impossible. The activities of many urine samples were so low that the error of replicate analyses in some cases reached 20 to 30% standard deviation (S. D.).<sup>5</sup> The error in the  $\text{Ca}^{45}$  analyses of replicate ash solutions prepared from stool specimens was always less than 10% S. D.

### EXPERIMENTAL RESULTS

The average results of the analyses for content of Ca and  $\text{Ca}^{45}$  of the serum, urine and feces of each individual are presented in table 2. The group averages are shown in table 3.

Inspection of the serum data revealed no striking group differences. This was confirmed by statistical evaluation (see below). The specific activity of the serum averaged 0.023% of the ingested  $\text{Ca}^{45}$  per milligram of serum Ca at 2.5 hours following the ingestion of the test meal. This figure is lower than the comparable uptake figure (0.028% mg) reported by us previously for somewhat older boys (Bronner et al., '54; see table 4).

<sup>5</sup> Percent standard deviation:

$$\frac{100(\sum x_i^2 - \bar{x}_i \sum x_i)}{(\sum x_i (n-1))^2}$$

lesterol values. The average fat intake of the older group was  $75 \pm 3$  gm per day and for the younger group was  $76 \pm 3$  gm per day. This consisted of about 55 gm of animal fat from butter, milk, meat, bacon and eggs. The remainder was vegetable fat including salad oils, peanut butter and hydrogenated fats used in frying and in making pies, cakes and biscuits. The average cholesterol intake of the older group was  $472 \pm 17$  mg per day and of the younger group,  $447 \pm$

TABLE 1

*Average daily nutrient intakes of 15 women with an average age of 31 years and of 14 women with an average age of 64 years, living in an institution*

NUTRIENT	YOUNGER GROUP Mean $\pm$ S.E. <sup>1</sup>	OLDER GROUP Mean $\pm$ S.E. <sup>1</sup>
Calories	2018 $\pm$ 78	2003 $\pm$ 44
Protein, gm	72 $\pm$ 3	72 $\pm$ 2
Fat, gm	76 $\pm$ 3	75 $\pm$ 2
Carbohydrate, gm	253 $\pm$ 9	251 $\pm$ 4
Calcium, mg	1002 $\pm$ 82	997 $\pm$ 75
Iron, mg	11.6 $\pm$ 0.4	11.7 $\pm$ 0.2
Vitamin A, I.U.	7972 $\pm$ 728	7054 $\pm$ 467
Thiamine, mg	1.22 $\pm$ 0.05	1.22 $\pm$ 0.02
Riboflavin, mg	1.91 $\pm$ 0.13	1.83 $\pm$ 0.10
Niacin, mg	13.7 $\pm$ 0.5	13.3 $\pm$ 0.2
Ascorbic acid, mg	32 $\pm$ 1	32 $\pm$ 1
Cholesterol, mg	447 $\pm$ 36	472 $\pm$ 17

<sup>1</sup> Standard error.

36 mg per day. Correlations between serum cholesterol and fat intake, and serum cholesterol and cholesterol intake were not significant.

The younger women were found to be, on the average, 7% overweight and the older women, 20% overweight. No significant correlation appeared between percentage deviation from desirable weight and serum cholesterol. The younger women lost, on the average, 0.8 lb during the study. The greatest loss was 8 lb and the greatest gain was 6 lb. The older women had an average weight change of zero, with a range from  $-7$  lb to  $+7$  lb.

Both the urinary and fecal excretions of Ca (table 2) reveal wide intra- and interindividual differences. Interindividual differences are common, especially in growing subjects. Intraindividual differences can also be expected in these boys,

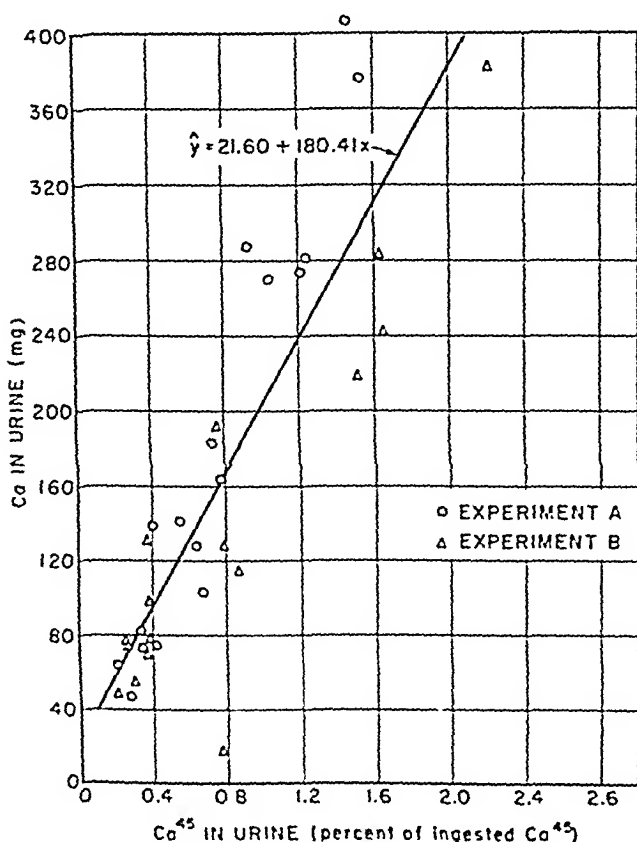


Fig. 1  $\text{Ca}^{45}$  output in urine as a function of the output of calcium, using pooled 72-hour samples.

particularly when experiments cannot be conducted under conditions which permit absolute control of intake.

Figure 1 shows the highly significant linear relationship observed between the output of Ca and  $\text{Ca}^{45}$  in the urine. The slope of this regression line is the specific activity and is independent of variations in the Ca output in the urine.



A slight rise in serum cholesterol with rise in serum ascorbic acid was noticed in the older group, but not in the younger group.

The correlation between serum cholesterol and percentage deviation from desirable weight was not significant.

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Therefore the measurements of total  $\text{Ca}^{45}$  output were used in the evaluation of the fecal data.<sup>9</sup>

Table 2 shows that total Ca output had a wider range in the urine than in the feces. Excretion in the urine is a more direct measure of Ca metabolism than is excretion in the stool. On the other hand, fecal output reflected more directly the relatively uniform intake of Ca in the diet of these subjects.

#### STATISTICAL ANALYSIS

Paired comparison tests (Snedecor, '46) were done on the data for specific activity and output to see how differently the two diet groups had disposed of their  $\text{Ca}^{45}$ . Table 3 summarizes the results of these tests.

Because the data for serum were incomplete in experiment A, the paired comparison test was supplemented with a group comparison. This test again showed no significant effect attributable to the presence of phytate in the diet.

In general, the specific activity of the urines paralleled that of the sera. However, the mean specific activity of the urines, but not of the sera, of subjects 28 to 35 was higher after oatmeal than after farina. This difference was significant on a 10% probability level, but is not likely to have been caused by phytate, which would have depressed  $\text{Ca}^{45}$  uptake and, therefore, decreased the  $\text{Ca}^{45}$  output in the urine. A similar reversal was also observed for the data on fecal output (table 3).

Analysis of the data for the output of  $\text{Ca}^{45}$  in the feces showed that subjects 19 to 27 excreted significantly more  $\text{Ca}^{45}$  following the oatmeal than following the farina breakfast. On the other hand, subjects 28 to 35 excreted significantly more  $\text{Ca}^{45}$  after the farina than after the oatmeal breakfast.

<sup>9</sup> It has been shown (Brenner et al., '56) that the total quantity of absorbed Ca which is excreted within 5 days of its ingestion is relatively small and can probably be neglected in a first approximation.



The results obtained in this and in the preceding study of this series (Bronner et al., '54) are compared in table 4 which shows that phytate lowered Ca absorption at the lower, but not at the higher, level of Ca intake. Table 4 also shows that under comparable conditions an increase in the Ca intake caused a decrease in the *percentage* of Ca absorbed. Hansard and Plumlee ('54) have presented similar, but more extensive, data for rats and have come to a similar conclusion (also Holtz,

TABLE 4  
*Effect of two levels of calcium and of phytate intake on the distribution of ingested  $\text{Ca}^{45}$*

PHYTATE INTAKE		CALCIUM INTAKE	
		86 mg <sup>1</sup>	239 mg
0.1 (Oatmeal)	<i>pm</i>		
	Serum <sup>2</sup>	0.028 <sup>3</sup>	0.023
	Urine <sup>4</sup>	0.0058	0.0048
0.0 (Farina)	Feces <sup>5</sup>	44.5	54.2
	Serum <sup>2</sup>	0.046 <sup>3</sup>	0.023
	Urine <sup>4</sup>	0.0065	0.0045
	Feces <sup>5</sup>	24.2	46.6

<sup>1</sup> Data adapted from Bronner et al. ('54).

<sup>2</sup> Percentage of ingested  $\text{Ca}^{45}$ /mg serum Ca at 2.5 hours following test meal.

<sup>3</sup> Serum data adjusted to mean body weight of 38.8 kg. Unadjusted figures: 0.020%/mg (oatmeal), 0.034%/mg (farina).

<sup>4</sup> Percentage of ingested  $\text{Ca}^{45}$  per milligram urinary Ca in 72-hour urine pool.

<sup>5</sup> Percentage of ingested  $\text{Ca}^{45}$  in 120-hour feces pool.

Popper and Silberman, '47). Recently Brine and Johnston ('55) analyzed the data in the literature and reported that the percentage of calcium absorbed by adults decreases when their intake increases.

The experiments reported here and previously (Bronner et al., '54), were designed to answer two related questions: (a) whether less Ca would be taken up from a phytate-rich than from a phytate-poor meal; and (b) what significance this might have in terms of practical nutrition. Table 4 shows

it was found that feeding a purified diet in which only the protein (casein) had been fumigated resulted in a similar severe growth inhibition. The present paper describes some of the preliminary experiments and those which led to the finding that the histidine and methionine of casein are affected by ETO fumigation.

#### EXPERIMENTAL

The albino rats employed in these studies were 21- to 24-day-old weanlings from our stock colony. The colony originated from the Holtzman strain. The rats were caged individually in wire-bottom cages and were supplied with fresh diet and water daily. The animals were weighed weekly or at the end of the experiment. The young rats were randomized among the experimental groups according to litter, sex and weight.

The stock diet used had the following percentage composition: ground wheat, 56.5; casein,<sup>2</sup> 12.0; meat scrap, 10.0; skim milk powder, 8.0; hydrogenated vegetable oil,<sup>3</sup> 5.0; molasses, 5.0; alfalfa meal, 2.0; vitamin A and D concentrate<sup>4</sup> (5,000 and 625 USP units of vitamins A and D<sub>2</sub> respectively per gram), 1.0; and salt, 0.5. Purified diet 3 contained the following (in per cent): sucrose, 73.0; casein, 18.0; B vitamins in sucrose, 5.0; and minerals (Salmon, '47), 4.0. Purified diet 4 was similar except that the casein was reduced to 9%, the sucrose increased to 72%, and 10% of hydrogenated vegetable oil was included. The B-vitamin supplement was made up to provide the following per kilogram of diet: 2 gm choline-Cl; 200 mg inositol; 50 mg niacin; 20 mg calcium pantothenate; 10 mg riboflavin; 5 mg pyridoxine-HCl; and 5 mg thiamine-HCl. The purified diets were further supplemented per kilogram with 50 mg alpha-tocopherol, 5 mg beta-carotene, and

<sup>2</sup> The casein was Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>3</sup> Crisco.

<sup>4</sup> Quadrex.

$\text{Ca}^{45}$  of each subgroup was affected significantly by changing the cereal of the test breakfast from oatmeal to farina. However, the direction of change was opposite for each subgroup. When the results of the fecal output of the two subgroups were pooled, the difference between the two test breakfasts was no longer significant.

4. The percentage of absorbed Ca decreased as the Ca intake increased.

5. It is concluded that phytates do not exert a significant effect on  $\text{Ca}^{45}$  absorption when the meal provides 239 mg of Ca and when the phytic P intake is 80 mg. Because this ratio of Ca to phytic P is typical of diets in the United States, it may be concluded that food phytates are of no nutritional concern in this country.

#### ACKNOWLEDGMENT

It is a pleasure to record our thanks to Mrs. Alice J. Chiu and Mrs. Dorothy Kuchta for technical assistance; to Dr. Malcolm J. Farrell, Superintendent, and to the staff and personnel of the Walter E. Fernald State School, Waverley, Massachusetts, for their help; and to the boys for their willing cooperation.

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this time the rats were moribund and were sacrificed for autopsy. Aside from a severe depletion of depot fat, no specific lesions could be detected, even upon microscopic examination of the major tissues. Control animals fed the same diet without prior fumigation performed normally over the experimental period.

When the stock diet, fumigated for 6 hours, was fed to 6 older rats (160 to 240 gm), they lost an average of  $10.6 \pm 7.3$  gm the first week and then gained slowly (av.,  $21.3 \pm 9.3$  gm) the three following weeks. No indication of neural damage was observed in these older rats. On autopsy, after 25 days on the fumigated diet, the contents of the large intestine

TABLE 1

*Thiamine stimulation of the growth of weanling rats fed a thiamine-deficient basal diet (diet 2) before and after fumigation with ETO*

DIET	NO. OF RATS	AV. WT. GAIN OVER DAYS	
		1-7	8-32
		gm	gm
Basal + thiamine-HCl	4	$15.0 \pm 1.4$	$81.7 \pm 19.3$
Fumigated basal + thiamine-HCl	4	$-6.5 \pm 2.6$	$17.3 \pm 5.6$
Basal	3	$12.3 \pm 1.6$	$-27.3 \pm 8.8$
Fumigated basal <sup>1</sup>	3	$-8.0 \pm 1.0$	$-26.7 \pm 5.0$

<sup>1</sup> Fumigated 14 hours.

and cecum was found to be more fluid, richer in mucous, and lighter in color than similar contents from control rats.

The growth inhibition resulting from feeding a fumigated thiamine-deficient diet (diet 2) supplemented with non-treated thiamine is apparent from the data in table 1. Only about one-third of the growth depression resulting from fumigating this diet was reversed by bi-weekly subcutaneous injections of 0.4 mg of thiamine-HCl per 100 gm of food intake. Feeding the thiamine-deficient diet without supplementation resulted in a 59% greater loss in body weight after 32 days when the diet had been previously fumigated with ETO.

Table 2 presents the results of feeding a purified diet in which only the protein (casein) had been fumigated. The

## TRYPTOPHAN-NIACIN METABOLISM IN ALLOXAN DIABETIC RATS <sup>1</sup>

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### TWO FIGURES

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It has been shown that the rat's dietary requirement for niacin could be altered by changing the type of carbohydrate in the diet (Hundley, '49). Since carbohydrate metabolism is modified in diabetic animals, it appeared of interest to determine whether the presence of a diabetic state would influence the niacin requirement of rats. It was reported that the urinary excretion of N<sup>1</sup>-methylnicotinamide (NMN) following test doses of nicotinamide was significantly lower in human diabetics than in control subjects (Lossy et al., '51), although later studies by Goldsmith et al. ('55) did not confirm this finding. Xanthurenic acid and 3-hydroxykynurenine were found in the urine of diabetic patients by Kotake and Tani ('53). Rosen et al. ('55) observed increased urinary xanthurenic acid in diabetics following oral tryptophan, indicating disturbances in tryptophan metabolism.

Reported here are experiments designed to study the effect of alloxan diabetes upon the ability of the rat to produce and excrete NMN following administration of various precursors.

<sup>1</sup> A preliminary report of this work has been published (McDaniel et al., '55).

<sup>2</sup> The American Cancer Society, New York.



could improve growth when used as a supplement in a fumigated casein diet. The results of the first experiment are shown in table 3. Phase 1 (1 to 14 days) indicates the growth obtained when the proportion of fumigated casein to untreated casein in the diet is progressively increased. All diets in this phase contained a total of 9% of casein, but growth was inhibited progressively as the proportion of fumigated casein was increased.

TABLE 3

*Stimulatory effect of some amino acids on the growth of weanling rats fed a purified basal diet (diet 4) containing graded levels of casein fumigated for 24 hours. (4 rats per treatment)*

GROUP	CASEIN IN DIET		AVERAGE WT. GAIN OVER DAYS			
	Fumi- gated	Un- treated	1-14	15-21 <sup>1</sup>	22-28 <sup>2</sup>	29-34 <sup>3</sup>
			gm	gm	gm	gm
1	0.0	9.0	19.5 ± 5.3	10.3 ± 2.6	9.0 ± 2.1	...
2	1.5	7.5	14.2 ± 1.5	18.3 ± 1.3	12.8 ± 3.6	...
3	3.0	6.0	9.0 ± 1.8	20.8 ± 4.1	13.8 ± 2.9	...
4	4.5	4.5	6.2 ± 3.3	14.3 ± 5.3	22.5 ± 4.4	...
5	6.0	3.0	1.8 ± 1.5	1.2 ± 1.0	3.8 ± 1.1	19.8 ± 8.3
6	7.5	1.5	— 2.2 ± 1.5	— 3.8 ± 1.5	1.0 ± 0.0	17.5 ± 5.2
7	9.0	0.0	— 3.8 ± 1.7	— 5.3 ± 0.5	2.3 ± 0.5	14.8 ± 1.7

<sup>1</sup> Diet supplemented with methionine, cystine and threonine.

<sup>2</sup> Diet further supplemented as follows: group 4, histidine and arginine; group 5, isoleucine and lysine; group 6, leucine and tryptophan; and group 7, valine and phenylalanine.

<sup>3</sup> Diet supplemented with methionine, cystine, threonine, histidine and arginine.

During the third week (15 to 21 days) all the diets containing fumigated casein were supplemented with the three indicated amino acids at the level at which these amino acids would be expected (Block and Bolling, '45) in the fumigated casein component of the diet had it not been fumigated. Supplementation in subsequent phases of this experiment and similar experiments was controlled likewise. Thus the diet with 9% of ETO-fumigated casein received three times the supplementation of the 3% treated casein diet.

In addition to the stock diet, three niacin-deficient purified diets were used in these experiments. Diet 9221 contained 80% sucrose, 8% vitamin-free casein, 8% hydrogenated cottonseed oil<sup>\*</sup> and 4% Wesson ('32) salt mixture. Diet 9260 contained 75.85% sucrose, 9% vitamin-free casein, 3% gelatin, 0.15% L-cystine, 8% hydrogenated cottonseed oil and 4% Wesson salt mixture. Diet 9276 was similar to diet 9221, except that sucrose was replaced by fructose. Incorporated into each 100 gm of the purified diets were 1 mg each of thiamine HCl and pyridoxine HCl, 4 mg calcium pantothenate, 2 mg riboflavin, 1.25 mg folic acid, 200 mg choline Cl, 1  $\mu$ g biotin, 0.4 mg menadione, 5 mg  $\alpha$ -tocopherol acetate, 12000 USP units vitamin A and 2500 USP units vitamin D. In addition 12.5  $\mu$ g vitamin B<sub>12</sub> were added to diets 9221 and 9276.

#### RESULTS

In preliminary experiments it was observed that alloxan diabetic rats excreted lower amounts of NMN in the urine than did non-diabetic rats on similar diets. Administration of niacinamide or niacin resulted in marked increases in NMN in both normal and diabetic rats, but administration of tryptophan, which has been shown to be a precursor of NMN in the rat (Rosen et al., '46; Hundley and Bond, '49), produced marked increases only with non-diabetic rats. The low conversion of tryptophan to NMN in diabetic rats was observed whether the tryptophan was given with the diet, by stomach tube, or by intraperitoneal injection, and whether L- or DL-tryptophan was used (table 1).

It had been observed in earlier experiments that urinary NMN values often were higher during periods of fasting or limited food intake than during periods in which food was consumed ad libitum. Food intake of diabetic animals was much greater than for non-diabetics. However, it was shown by paired feeding and by fasting that the abnormally low NMN values observed for the diabetic rats given tryptophan were not the result of excessive food intake (table 2).

\* Crisco.

levels, the growth observed was fully comparable with that on an untreated casein diet. It would appear that if any other essential amino acid is affected when casein is fumigated as described it must be one which is not growth limiting under the conditions of this experiment.

TABLE 5

*Ability of histidine and methionine to stimulate growth of weanling rats fed a purified diet (diet 4) containing 9% ETO-fumigated casein<sup>1</sup>  
(6 rats per treatment)*

PROTEIN SOURCE	SUPPLEMENT TO DIET		AV. WT. GAIN, 21 DAYS
	L-histidine	DL-methionine	
	%	%	gm
Unfumigated casein	0.279	0.315	48.8 ± 6.4
Fumigated casein	0.279	0.315	39.8 ± 5.6
Unfumigated casein	....	....	30.5 ± 4.5
Fumigated casein	....	....	2.6 ± 1.5
Fumigated casein	0.279	....	2.2 ± 1.6
Fumigated casein	...	0.315	-1.4 ± 1.7

<sup>1</sup> Fumigated 24 hours.

TABLE 6

*Growth inhibition of weanling rats fed a purified basal diet (diet 4) containing 9% casein variably fumigated with ETO (8 rats per treatment)*

DURATION OF ETO FUMIGATION OF CASEIN	AV. WT. GAIN, 21 DAYS
	gm
0 min.	27.7 ± 7.3
15 min.	25.0 ± 5.3
30 min.	19.7 ± 7.0
1 hr.	19.7 ± 7.6
4 hr.	6.3 ± 2.2
24 hr.	-8.7 ± 1.6

The effect of the duration of the ETO fumigation of casein upon its biological value is seen in table 6. Growth depression was severe after 4 hours of fumigation, but maximum protein damage was not achieved until fumigation had proceeded for much longer periods, possibly 24 hours, or more.

Microbiological assay for histidine and methionine in 24-hour ETO-fumigated and non-fumigated casein samples indi-

The possibility that glucose or other metabolites in diabetic urine might interfere with the determination of NMN was investigated. NMN was determined on urine from normal rats supplemented with tryptophan, and on this urine diluted with urine from diabetic rats containing known amounts of glucose, and after addition of known amounts of C. P. glucose to normal urines. It was observed that the amount of NMN determined was reduced both by dilution with diabetic urine and by addition of glucose. The reduction caused by the diabetic urine appeared to be due entirely to the presence of glucose. Urinary NMN values for normal rats given tryptophan were reduced by about 25% when measured in the presence of diabetic urines (sufficient diabetic urine was added to approximate a rat excreting 8 gm of glucose per day). An equivalent amount of C. P. glucose added to the normal urine resulted in essentially the same reduction. When the amount of glucose was doubled (equivalent to a rat excreting 16 gm of glucose per day), the NMN determined was reduced by about 40%. Although glucose in amounts often found in severe diabetes may lower the NMN value by as much as 40%, this reduction is not of sufficient magnitude to account for the very low NMN values observed following administration of tryptophan to diabetic rats. Furthermore, as is shown in tables 1 and 2, during periods of fasting when no glucose is present in diabetic urine, the apparent conversion of tryptophan to NMN is still abnormally low. To demonstrate further that the low NMN values observed for diabetics were not due to interfering substances in the urine, it was shown that NMN resulting from administration of niacinamide, niacin and NMN could be determined readily even in diabetic urines (table 1).

In earlier studies a marked individual variation was observed in the levels of urinary NMN among rats on similar diets either with or without supplementary niacin or tryptophan (Hundley, '47). In view of this known individual variation and to eliminate the possibility that the differences observed in the present experiments were merely the result of

The effect of ETO on proteins other than casein remains to be determined. There is no indication to date that the lability of protein histidine and methionine is a general phenomenon. It is interesting to speculate, however, that ETO-protein (enzyme) reactions are involved in the lethal action of the fumigant on microorganisms.

Some reactions of ETO with protein have been described earlier (Fraenkel-Conrat, '44). ETO was found to react with most of the available reactive groups, namely, carboxyl, amino, sulphydryl and phenol groups. The reactions were studied only in aqueous solution, however, and their application to the conditions described in this paper remains to be investigated.

#### SUMMARY

Weanling rats failed to grow when fed a purified diet containing 9 or 18% of casein as the only protein source when this casein had been previously fumigated with ethylene oxide. A histidine and methionine supplement was active in reversing this inhibition. Only 29% of the histidine and 44% of the methionine of casein appeared to be available to the bacterium, *Lactobacillus mesenteroides* after 24 hours of fumigation of the intact protein with ethylene oxide.

#### ACKNOWLEDGMENTS

The authors are indebted to Dr. J. R. Rooney, II, Animal Pathology Section, Virginia Agricultural Experiment Station, Blacksburg, Virginia, for performing the gross and histological examinations of the sacrificed animals, to Mr. Howard Bakerman, of the Laboratory of Biochemistry and Nutrition, National Institutes of Health, Bethesda, Maryland, for the microbiological assays, and to Merck and Company, Rahway, New Jersey, for the B vitamins used in these studies.

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It was observed that the rate of response to insulin (with respect to conversion of tryptophan to NMN) varied in different rats, even though the water intake and urinary glucose of all rats tested decreased rapidly after the insulin was started. In one such rat, which also converted abnormally low amounts of tryptophan to NMN, the response to insulin was much more rapid, with NMN increasing to a normal level

## EFFECT OF INSULIN IN DIABETIC RATS

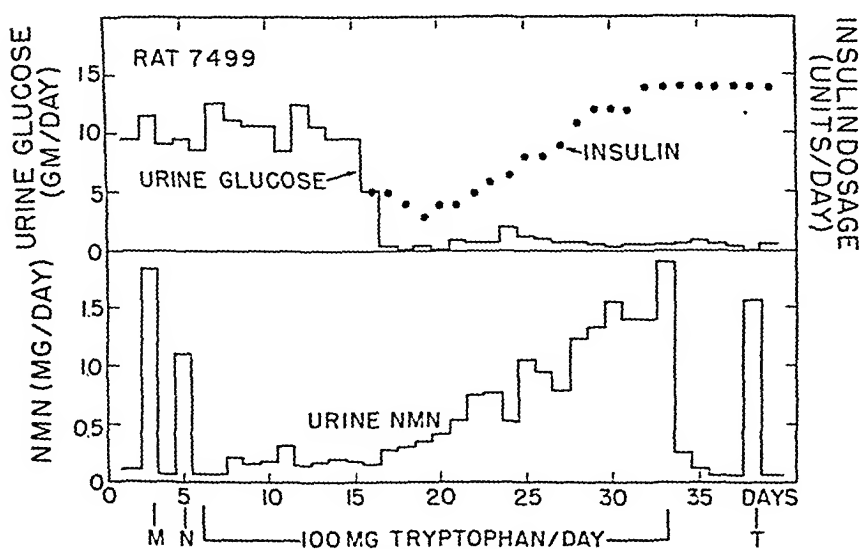


Fig. 1 Effect of insulin on conversion of tryptophan to NMN in diabetic rat. Diet 9221. T = 100 mg tryptophan; N = 3 mg niacinamide; M = 3 mg N<sup>1</sup>-methyl-nicotinamide.

Niacinamide, NMN, and insulin were given intraperitoneally. Single doses of tryptophan were given by stomach tube, and added to the diet for daily feeding.

within 24 hours after the insulin was started. For this rat the insulin was discontinued after 4 days. The conversion of tryptophan to NMN remained relatively constant and in the normal range for 8 days then decreased gradually to a diabetic level in about 16 days after the insulin was stopped. In view of these observations further studies were made to determine the effect of insulin on the conversion of tryptophan and



and by diabetic humans (Craig et al., '51; Miller et al., '52). Sarett and Snipper ('54) have shown that alloxan diabetic rats fed fructose diets consume much less water and excrete less carbohydrate in the urine than do rats fed glucose diets. In the present experiments substitution of fructose for sucrose

TABLE 3

*Effects of dietary fructose in alloxan diabetic rats<sup>1</sup>*

CATEGORY OF INTEREST	DIET 9221 (Sucrose)	DIET 9267 (Fructose)
Basal urinary NMN (mg/day)	0.08 (0.06-0.12)	0.07 (0.07)
Urinary NMN/100 mg tryp (mg/day)	0.22 (0.17-0.26)	0.18 (0.13-0.24)
Urinary glucose (gm/day)	11.1 (8.0-14.9)	7.1 (6.8-8.5)
Water intake (ml/day)	155 (135-194)	107 (94-123)

<sup>1</sup> Three diabetic rats were used in this experiment. Values in the second column were obtained from the same three rats after 8 to 24 days of fructose feeding. Ranges of values are shown within the parentheses.

### EFFECT OF TRYPTOPHAN ON BLOOD GLUCOSE CURVES

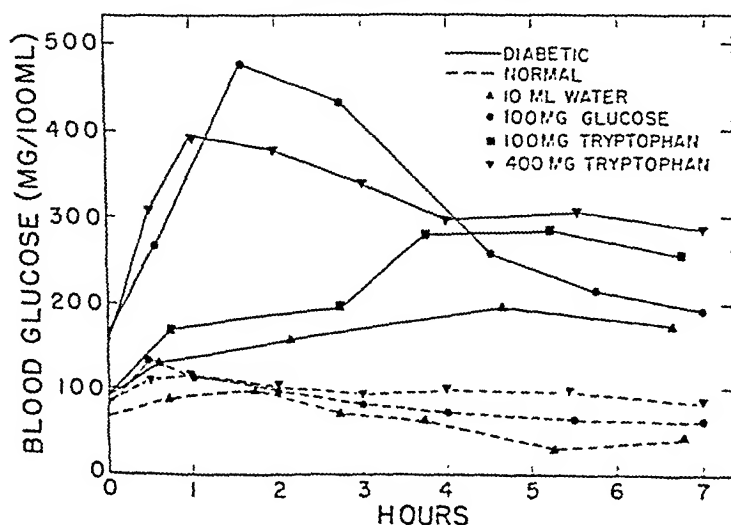


Fig. 2 Effect of tryptophan on blood glucose curves. Both rats on diet 9260. Glucose and tryptophan were given by stomach tube following a 16-hour fast.



Dick ('52) reported that a factor present in two different types of forage was involved in the copper-molybdenum imbalance in sheep, and in subsequent studies ('53a) identified this factor as inorganic sulfate. Results of the additions of inorganic sulfate to high-molybdenum rations for sheep have been further investigated by Dick ('53b, '54) who reported that the addition of sulfate to the high copper-molybdenum-containing ration increased the storage of copper in the liver. Furthermore, at a constant level of molybdenum supplementation liver copper levels increased as the level of sulfate increased. Similar results were observed when the blood copper concentration was considered. Observations of the fleece character of these sheep by Dick ('54) led him to conclude that these sheep were copper deficient while blood and liver copper levels were elevated.

The present report summarizes studies that illustrate the beneficial effects of dietary sulfate in molybdenum-fed rats. Data are also presented on the effect of varying sulfate and molybdenum levels in the diet on copper and molybdenum concentration in blood and liver.

#### EXPERIMENTAL

Equal numbers of male and female 21-day-old albino rats (35 to 50 gm) were used in these studies. The rats in experiment I (Holtzman strain) were from the stock colony of this laboratory while those in experiment II were obtained commercially.<sup>3</sup> All rats of the same sex were allotted at random to their respective treatments. The basal ration used in all experiments had the following percentage composition: sucrose 80.25, crude casein 12.0, cottonseed oil 5.0, low-sulfate salts 2.55, L-cystine 0.2.

Vitamins were added as follows (milligrams per kilogram of ration): choline chloride, 1,000; inositol, 100; calcium pantothenate, 20; niacin, 10; menadione, 10; thiamine·HCl, 5; riboflavin, 3; pyroxidine·HCl, 3; folic acid, 0.2; biotin, 0.1

<sup>3</sup> Holtzman Company, Madison, Wisconsin.

non-diabetics. However, when 400 mg of tryptophan were given, both xanthurenic acid and other metabolites increased markedly in the urine of all rats. Xanthurenic acid was increased much more in diabetics than in non-diabetics, while for the other metabolites the reverse was true.

TABLE 4

*Effect of tryptophan upon the urinary excretion of various tryptophan metabolites in diabetic and non-diabetic rats*<sup>1</sup>

RAT NO.	AFTER 100 MG TRYPTOPHAN <sup>2</sup>			AFTER 400 MG TRYPTOPHAN <sup>2</sup>		
	NMN	XA <sup>3</sup>	"Other" <sup>4</sup>	NMN	XA <sup>3</sup>	"Other" <sup>4</sup>
	<i>mg/day</i>			<i>mg/day</i>		
Diabetics	8690	0.05	14	0.23	32	70
	8709	0.10	2 <sup>5</sup>	0.52	50	62
	8675	0.18	19	0.88	65	32
Non-diabetics	8720	0.76	13	2.28	14	133
	8731	3.12	2 <sup>5</sup>	5.12	12	117
	8750	0.54	14	1.30	16	101

<sup>1</sup> Five diabetic and two non-diabetic rats not listed above were given 200 mg L-tryptophan. Average urinary values for the above compounds were as follows:

Diabetics 0.76 mg NMN; 25 mg XA; 13 mg "Other"

Non-diabetics 3.23 mg NMN; 8 mg XA; 70 mg "Other"

Unsupplemented rats (diabetics and non-diabetics) excreted averages of 3.5 mg/day of XA and 3.2 mg/day of "other" metabolites.

<sup>2</sup> Tryptophan was given by stomach tube during periods when animals were otherwise fasted. When 400 mg tryptophan were given it was necessary to use the more soluble L-form. No marked differences in the conversion of L- and DL-tryptophan to NMN have been observed.

<sup>3</sup> XA = xanthurenic acid.

<sup>4</sup> "Other" indicates compounds excreted in the urine which give a positive reaction in the Eckert ('43) test, and expressed as mg/day using tryptophan as a standard. Anthranilic acid, tryptophan, possibly kynurenine and other metabolites will react in this test.

<sup>5</sup> Values shown for xanthurenic acid (XA) following 100 mg tryptophan are averages of two diabetic and two non-diabetic rats not listed above.

## DISCUSSION

It is evident that in the alloxan diabetic rat there is some disturbance in the metabolism of tryptophan which results in a marked impairment in converting tryptophan to niacin and then to NMN. The results of the present study suggest that

TABLE 1

*The alleviation of molybdenum-induced rat growth inhibition with inorganic sulfate (22 rats per treatment, 5 replicates)*

DIET	AV. 6 WEEK BODY WEIGHT GAIN
	gm
Basal	109 ± 18 <sup>3</sup>
Basal + SO <sub>4</sub> <sup>1</sup>	112 ± 23
Basal + Mo <sup>2</sup>	60 ± 15
Basal + SO <sub>4</sub> + Mo	102 ± 27

<sup>1</sup> 2,000 p.p.m. SO<sub>4</sub> as 1:1 Na<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>SO<sub>4</sub>.

<sup>2</sup> 100 p.p.m. Mo as Na<sub>2</sub>MoO<sub>4</sub>.

<sup>3</sup>  $\sqrt{\frac{\sum(x)^2}{n-1}}$ .

TABLE 2

*Dietary molybdenum level and the effect of inorganic sulfate upon rat growth and blood and liver levels of molybdenum and copper*

(Experiment I, 4 rats/treatment)

LOT AND TREATMENT	AV. 6 WEEK GAIN	WHOLE BLOOD		LIVER (DRY, FAT-FREE)	
		Copper conc.	Molybdenum conc.	Copper conc.	Molybdenum conc.
	gm	μg/ml	μg/ml	μg/gm	μg/gm
1. Basal	78 ± 22	0.6	Trace	10.0 ± 1	1.6 ± 0.5
2. Basal + 75 p.p.m. Mo <sup>1</sup>	47 ± 11	4.0	13.4	16.5 ± 4	29.8 ± 1.7
3. Basal + 300 p.p.m. Mo	17 ± 8	6.5	13.4	38.0 ± 15	51.6 ± 12
4. Basal + 2,200 p.p.m. SO <sub>4</sub> <sup>2</sup>	76 ± 17	0.5	Trace	8.0 ± 1.4	1.4 ± 0.2
5. Basal + 75 p.p.m. Mo + 2,200 p.p.m. SO <sub>4</sub>	84 ± 22	2.5	5.7	12.3 ± 1	9.2 ± 1.6
6. Basal + 300 p.p.m. Mo + 2,200 p.p.m. SO <sub>4</sub>	53 ± 12	3.0	9.3	16.0 ± 1.6	19.0 ± 5.5

<sup>1</sup> As H<sub>2</sub>MoO<sub>4</sub>.

<sup>2</sup> As equimolar mixture of Na<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>SO<sub>4</sub>.

flavin deficiency in rats has been shown to cause abnormal tryptophan metabolism (Henderson et al., '51; Mason, '53) and also results in development of cataracts (Day et al., '31; Day and Langston, '34; Bourne and Pyke, '35). The cataracts resulting from riboflavin deficiency closely resembled those due to tryptophan deficiency, according to Albanese and Buschke ('42). Whether there is a relationship between abnormal tryptophan metabolism and development of cataracts remains to be determined.

It is of interest that for some diabetic rats, in which the conversion of tryptophan to NMN was low when 100 mg doses were given, increased amounts of NMN could be excreted if very large doses (400 mg) of tryptophan were given. This suggests that, although the mechanism for conversion appears to be greatly impaired, the real difficulty may result from shifts in the primary metabolic pathways of tryptophan rather than from the complete absence of the proper mechanisms. In addition, the marked difference between diabetics and non-diabetics, with respect to the relative amounts of urinary xanthurenic acid and other metabolites following large doses of tryptophan, also suggests modification of the pathways of tryptophan metabolism in diabetic rats.

The rate of response to insulin, with respect to conversion of tryptophan to NMN, varied with different rats. Although the water intake and urinary glucose decreased rapidly after insulin was started, the response with respect to NMN excretion ranged from one to 19 days. The delayed responses observed following administration or withdrawal of insulin suggest that the process which permits increased conversion of tryptophan to NMN may result indirectly from the action of insulin upon some other mechanism.

Data obtained by chromatographic and chemical procedures indicate that ability to excrete anthranilic acid and 3-hydroxyanthranilic acid in the urine following administration of tryptophan is markedly reduced in diabetic rats, and that kynurenic acid is present in the urine of both diabetic and non-diabetic rats. Tryptophan may be converted to niacin

of the femurs of these rats indicated a chondro-dystrophy of the epiphysial cartilages. The femurs of the rats in the other lots were normal when examined grossly and histologically. The percentage of femur ash was determined on the opposite

TABLE 3

*The effect of dietary inorganic sulfate level upon the molybdenum-induced rat growth inhibition and upon blood and liver levels of molybdenum and copper*

(Experiment II, 4 rats/treatment)

LOT AND TREATMENT	AV. 6 WEEK GAIN	WHOLE BLOOD		LIVER (DRY, FAT-FREE)	
		Copper conc.	Molybdenum conc.	Copper conc.	Molybdenum conc.
	gm	μg/ml	μg/ml	μg/gm	μg/gm
Basal	98 ± 10	0.6	Trace	10.0 ± 2	2.6 ± 1.4
Basal + 100 p.p.m. Mo <sup>1</sup>	47 ± 6	5.7	16.6	40.6 ± 15	48.3 ± 12
Basal + 400 p.p.m. SO <sub>4</sub> <sup>2</sup>	102 ± 14	0.45	Trace	10.6 ± 1	2.4 ± 1.1
Basal + 400 p.p.m. SO <sub>4</sub> + 100 p.p.m. Mo	66 ± 18	4.9	10.6	28.6 ± 5	20.4 ± 6
Basal + 800 p.p.m. SO <sub>4</sub>	80 ± 11	0.4	Trace	10.8 ± 2	2.3 ± 0.8
Basal + 800 p.p.m. SO <sub>4</sub> + 100 p.p.m. Mo	83 ± 26	3.8	9.9	22.2 ± 4	17.9 ± 4
Basal + 2,200 p.p.m. SO <sub>4</sub>	95 ± 25	0.5	Trace	10.6 ± 0.8	1.8 ± 0.6
Basal + 2,200 p.p.m. SO <sub>4</sub> + 100 p.p.m. Mo	100 ± 17	4.7	10.0	27.2 ± 2.5	19.0 ± 3
Basal + 3,300 p.p.m. SO <sub>4</sub>	95 ± 7	0.5	Trace	11.6 ± 2	1.6 ± 0.4
Basal + 3,300 p.p.m. SO <sub>4</sub> + 100 p.p.m. Mo	96 ± 28	3.8	7.6	23.8 ± 5	14.9 ± 3

<sup>1</sup> As Na<sub>2</sub>MoO<sub>4</sub>.

<sup>2</sup> As equimolar mixture of Na<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>SO<sub>4</sub>.

of a beneficial effect of dietary fructose in correcting the low conversion of tryptophan to NMN, indicates that conversion to glucose is not the entire explanation.

Some diabetic animals in which the conversion of tryptophan to NMN was greatly impaired did excrete increased amounts of NMN when very large doses of tryptophan (400 mg) were given, indicating that the defect may be due to changes in the primary metabolic pathways of tryptophan rather than to absence of the proper mechanisms.

Diabetics excreted much more xanthurenic acid than did non-diabetics, following large doses (200 to 400 mg) of tryptophan.

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denum. When 100 p.p.m. of molybdenum was added to the diet it appeared that a level of sulfate between 800 and 2,200 p.p.m. exerted its maximum growth-protective effect.

#### ACKNOWLEDGMENTS

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#### ADDENDUM

Since this manuscript was submitted, R. Van Reen and M. A. Williams have published results indicating that sulfur compounds alleviated the toxicity of molybdenum for the rat. *Archives of Biochem. Biophysics*, 63: 1 (1956).

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# THE EFFECT OF AGE ON THE LEVEL AND METABOLISM OF FLUORINE IN THE BONES OF THE FLUORIDATED RAT<sup>1,2</sup>

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Fluorine deposition in the skeleton may occur by means of actual incorporation into the bone salt molecule, or by ionic exchange as suggested by Klement ('37), Neuman et al. ('50) and Megirian and Hodge ('51). A growing animal would therefore incorporate fluorine by both means while the pre-formed bones of the adult would deposit skeletal fluorine in large measure only by ionic exchanges and periosteal growth. Exostoses or newly formed spicules of bone salts in cancellous bones under the influence of excess dietary fluorine would increase the fluorine content of the resulting bone deposits of the animal regardless of the age factor.

A reverse of the ionic exchange reaction apparently takes place in the reduction of the total fluorine content in the skeleton. For a reduction in the total skeletal fluorine to occur.

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<sup>2</sup> Submitted in part as a Ph.D. thesis by Russell F. Miller to the Graduate School of the University of Wisconsin.

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order to demonstrate that all subjects were capable of maintaining nitrogen equilibrium on an adequate diet and to establish a level of nitrogen intake for use throughout the experiment. In a transition period (three to 4 days) the items of food in the normal diet were gradually replaced by those of the semi-synthetic diet which was then fed throughout the remainder of the experiment. For the first 12 to 16 days on the semi-synthetic diet, lysine was supplied at the level found in 20 gm of egg protein by a so-called "complete" amino acid mixture. In series I and II, after it had been established that all subjects were in nitrogen balance on this regimen, the need for lysine was demonstrated by their inability to attain nitrogen equilibrium during 10 to 11 days when all of the lysine in the supplements was replaced by isonitrogenous amounts of glycine. Six different levels of lysine were fed successively in series I, and 4 in series II. The subjects in series II participated in a methionine study for a 30-day interval between the time the complete amino acid supplements were fed and the initial feeding of the lysine-deficient supplements.

In series III, the "complete" amino acid mixture was fed as before, but thereafter lysine and methionine were fed at levels which had appeared to be adequate in series I and II and in certain other studies by Reynolds ('56); the daily amino acid supplements supplied 300 mg of lysine, 250 mg of methionine and 480 mg of cystine for two periods of 4 days each. Other variations in procedure have been recorded elsewhere (Jones, '56).

*Subjects.* The subjects were women students or staff members 19 to 43 years of age who maintained their usual academic pursuits throughout the experiment (table 1). All were in normal health as determined by physical examinations at the Department of Student Health, University of Wisconsin. In series I and II, the subjects were housed in an apartment under the direction of a graduate dietitian, whereas in series III, they lived in dormitories or private homes, but ate all

animals were then transferred to the basal ration only (without added fluorine) for periods up to 300 days. The preliminary feeding period was for the purpose of rapidly fluoridating the skeletons of these rats of various ages. At the close of the fluoridating period rats from each age group were sacrificed, the femurs removed and analyzed for their fluorine content. Four rats were removed at intervals of 15, 30, 60, 90 and 120 days post-fluoridation and femur fluorine determined as indicated. Four rats (6 months age group) were sacrificed at 300 days.

The results obtained are summarized in table 1. The data indicate rapid and heavy fluorine deposition in the femurs of the three week age group to near the skeletal saturation levels (16,000 to 20,000 p.p.m.), while the 7 weeks (young adult) and 6 months old rats (adult) stored, respectively, approximately 50 and 25% as much fluorine as the weanlings.

The data also show clearly that the fluorine concentration and total fluorine of the femurs of the young dropped rapidly following the removal of the added dietary fluorine. These results are similar in pattern and magnitude to those obtained earlier by Miller and Phillips ('53), and they are in line with the observations reported by Savchuck and Armstrong ('51). The total fluorine content of the mature bone as indicated by milligrams of fluorine per femur did not decrease during the post-fluoridation period although there was a slight decrease in femur fluorine concentration, expressed as parts per million. This observation is believed to be the result of the dilution effect of new bone growth. It is remarkable that the fluorine varied only about 600 p.p.m. between the various lots at 120 days post-fluoridation.

### *Experiment 2*

This experiment was designed to determine the femur concentrations of fluorine in young rats under continuous exposure to 0.10% of dietary NaF from weaning up to periods of 18 weeks. Three groups of 18 white rats were selected and

TABLE 2

*Semi-synthetic diet exclusive of the nitrogen supplements*

ITEM	WEIGHT	ITEM	WEIGHT
	gm		gm
Applesauce, canned, sweetened	200	Sanka <sup>2</sup>	
Butter oil	43	Sucrose	180
Carrots, raw	25	Tomatoes, canned	100
Grape juice, canned	100	Wafers: <sup>3</sup>	1 recipe
Jelly	40	Butter oil	10
Lemon juice, canned	75	Cornstarch	50
Orange juice, frozen, reconstituted	100	Hemicellulose <sup>4</sup> (Mucilose flakes)	3
Peaches, canned, freestone	100	Salt	4
Peach syrup, canned	50	Sucrose	20
Pudding: <sup>1</sup>	1 recipe	Wesson oil	7
Butter oil	13	Water	57
Cornstarch	8	Baking powder — Mineral mixture <sup>5</sup>	9.4
Salt	1		
Sucrose	30		
Water	90		

<sup>1</sup> Basic recipe was obtained from Leverton ('53). Vanilla or peppermint pudding was prepared by adding one or two drops of the extract. Lime or lemon pudding was made by substituting 15 gm of the fresh juice (strained) for 15 gm of the water. Twenty grams of sucrose was replaced by brown sugar in the butterscotch pudding.

<sup>2</sup> Sanka was served at breakfast and dinner. Quantities were based on individual preference, but were constant for any given individual.

<sup>3</sup> Basic recipe was obtained from Leverton ('53).

<sup>4</sup> Mucilose Flakes, Winthrop-Stearns, Inc.

<sup>5</sup> The mixture contained 1.8 gm of mineral supplement and 7.6 gm of the baking powder. The composition of the baking powder and of the mineral supplements were given by Leverton et al. ('56).

diet used in series II and III is presented in table 2. In series I the diet contained an additional 100 gm of grape juice, 25 gm of lettuce and 100 gm of potato, or an additional 50 gm of potato. In series I the basal portion of the semi-synthetic diet supplied from 0.6 to 1.0 gm of nitrogen and from 0.10 to 0.25 gm lysine per day depending upon the foods which were included.<sup>5</sup> In series II and III the basal portion of the diet

<sup>5</sup> The results of the analysis of potatoes were variable; one variety yielded 4.7 mg of nitrogen and 1.41 mg of lysine, whereas a second contained 2.7 mg of nitrogen and 0.86 mg of lysine per gram of potato.

distributed as follows: lot 1, weanlings three weeks old; lot 2, young adults 9 weeks old; and lot 3, adults 5 months old. All rats were fed the basal ration used in experiment 1.

The results obtained from femur fluorine analyses (ashed basis) of the rats of the various ages show that the femurs of the young weanling rats were fully fluoridated (p.p.m.) after 6 weeks' exposure to the dietary fluorine, while the femur fluorine concentration of the two groups of adult rats continued to increase for the entire period of 18 weeks (table 1). At the close of the experimental period the young adult, 9 weeks old rat femurs averaged 80% as much fluorine in parts per million as the weanling rats. Likewise the adult rats (5 months old at the beginning of the experiment) had femur fluorine concentrations approximately half those of the weanling rat. The total fluorine content of the femurs increased with the length of the exposure period. The increase, which may be accounted for by the growth of the bone, cannot be completely explained on this basis since the adult group, lot 3, would seem to have started with a fully mature femur at 5 months of age and yet the total fluorine deposited in the femurs between 12 and 18 weeks was greater than during earlier periods.

### *Experiment 3*

This experiment was conducted to determine the effect of re-exposure to dietary fluorine upon the deposition of fluorine in the femur, as related to age when first exposed. Thirty-two white rats representing one of three age levels were used per lot. Lot 1 was composed of weanlings three weeks old, lot 2, young adults 9 weeks old, and lot 3, adults 5 months of age when they were first exposed to added dietary NaF (0.10%). The ration used was the same as that used in the previous experiments. Each lot started with 26 rats for the first fluoridation period. In addition, 6 unfluoridated rats served as controls and were fed the basal ration only until their lot mates were ready for refluoridation. Four of these were then fluoridated and thus received only a single 6 weeks exposure

the total nitrogen content was maintained constant by a suitable reduction of the glycine.

A mixture of all the amino acids except cystine and tyrosine was ball-milled over night and sieved. Any material which did not readily pass through the sieve was ground in a mortar

TABLE 3

*Individual daily allotments of the amino acids and diammonium citrate*

AMINO ACID	AMOUNT	NITROGEN
	gm	gm
<i>Solution supplement:</i>		
L-Arginine hydrochloride	1.549	0.412
L-Histidine hydrochloride	0.519	0.114
DL-Isoleucine	3.200	0.342
L-Leucine	1.840	0.197
L-Lysine hydrochloride <sup>1</sup>	1.800	0.276
L-Methionine	0.820	0.077
L-Phenylalanine	1.260	0.107
L-Threonine	0.930	0.115
L-Tryptophan	0.300	0.041
L-Valine	1.460	0.175
Glycine	21.473	4.009
SUBTOTAL	35.201	5.865
Diammonium citrate	32.357	4.009
<i>Dry powder supplement:</i>		
L-Cystine	0.480	0.056
L-Tyrosine	0.900	0.070
SUBTOTAL	1.380	0.126
TOTAL		10.000

<sup>1</sup> Since this compound was only 95% pure, 1.895 gm was used.

and the entire batch was returned to the ball mill for additional mixing. One-fourth of the day's quota of the mixture was fed at breakfast, and three-eighths each at luncheon and dinner. The precise amounts were weighed for each subject for each meal. Sixty grams of sugar were added to each portion and sufficient hot distilled water was used to put all of the amino acids in solution. A diammonium citrate solution

TABLE 2

*The effect of age upon femur fluorine deposition, mobilization and re-fluoridation in the rat*

TREATMENT	NUMBER ANIMALS PER LOT	LOT 1 (weanling)		LOT 2 (9 wks.)		LOT 3 (5 mo.)	
		FLUORINE		FLUORINE		FLUORINE	
		mg/femur	p.p.m.	mg/femur	p.p.m.	mg/femur	p.p.m.
1st fluoridation	6	1.58 ± 0.14	15360 ± 900	1.54 ± 0.19	7700 ± 950	1.28 ± 0.18	4900 ± 690
60 day recovery - F	4	1.14 ± 0.09	5250 ± 670	1.50 ± 0.15	5900 ± 280	1.16 ± 0.13	4070 ± 460
Re-fluoridation (after 60 day - F)	4	1.90 ± 0.16	7780 ± 870	1.93 ± 0.15	7000 ± 510	1.36 ± 0.15	4700 ± 290
120 day recovery - F	4	1.13 ± 0.09	4370 ± 470	1.36 ± 0.04	5000 ± 370	1.18 ± 0.16	4065 ± 390
Re-fluoridation (after 120 day - F)	4	1.90 ± 0.08	7520 ± 220	2.00 ± 0.39	6950 ± 620	1.52 ± 0.15	5410 ± 410
Controls* fluoridated at approximately		18 wks		24 wks		35 wks	
	4	0.91 ± 0.06	3640 ± 150	0.78 ± 0.03	3090 ± 80	0.95 ± 0.17	3200 ± 480
Controls* fluoridated at approximately		24 wks		32 wks		43 wks	
	4	0.98 ± 0.15	3560 ± 330	0.98 ± 0.01	3340 ± 460	0.98 ± 0.04	2950 ± 260

\* Unfluoridated rats of similar ages had 0.2 mg F/femur and concentration of less than 600 p.p.m.



added to cover. After the gases had been exhausted from the samples by holding the jars in a hot-water bath for about 12 hours, they were autoclaved for three hours at 15 pounds of pressure, cooled, weighed, and sampled for analysis.

The nitrogen contents of the amino acid mixtures and adjustment solutions and of the food, urine and fecal samples were determined by a boric acid modification of the Kjeldahl method (Scales and Harrison, '20). The lysine contents of the foods and amino acid supplements were determined by microbiological assay (Jones, '56). Creatinine determinations were made on the daily urine samples with either the Peters ('42) or the Klett-Summerson adaptations of the Folin ('14) method. The constancy of the creatinine concentration was considered an indication of the completeness of collection of the daily urines.

#### RESULTS AND DISCUSSION

In agreement with the observations of others (Rose, Coon and Lambert, '54; Pratt et al., '55), a larger caloric intake was required to maintain the weights of the subjects on the semi-synthetic regimen, wherein most of the nitrogen was supplied by amino acids and diammonium citrate, than on the normal diet of natural foods. On the normal diet the mean daily caloric intakes of the individual subjects ranged from 1540 to 2115 with a mean of 1974; whereas, on the semi-synthetic regimen, they varied from 1761 to 2585 with a mean of 2286. The low values were for a relatively small subject. Expressed as calories per kilogram of body weight, the mean values for the subjects on the normal diet ranged from 28.3 to 36.0 with a mean of 32.8. The comparable figures for the semi-synthetic diet were 32.7 to 43.0 with a mean of 37.6 Cal. per kilogram.

Detailed metabolism data for a representative subject are summarized in table 4 which demonstrates the experimental plan and shows the fluctuations associated with this type of study. For this subject the mean daily nitrogen balances of the periods which supplied 0.10, 0.18, 0.22, 0.25 and 0.64 mg

maturation. The explanation of these observations is not clear at the present time.

#### SUMMARY

An effect of age upon the deposition and retention of femur fluorine has been demonstrated. Fluorine was deposited in greater amounts in the femur of the young rat than in that of the mature rat; however, both the young and mature rat femurs continued to concentrate fluorine progressively with time. Femur fluorine in the rat was mobilized from this bone during periods of low fluorine intake. Again age affected the rate and extent of such femur  $F^-$  catabolism. The mobilization of femur fluorine of the weanling rat accompanied by an increase in age caused bone changes in the femur which closed a portion of the available fluorine deposition sites to subsequent re-fluoridation.

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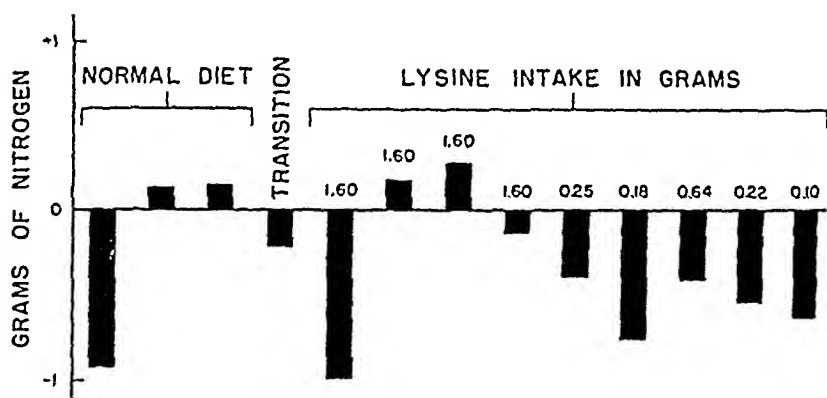


Fig. 1 The nitrogen balances of a representative subject (no. 8) at different levels of lysine intake, in sequence used.

TABLE 5

*Summary of nitrogen balances at different levels of lysine intake*

SUBJECT	MEAN DAILY NITROGEN BALANCE ON INDICATED DAILY INTAKE OF LYSINE, GM							
	0.10	0.18	0.22	0.25	0.40	0.50	0.61	1.60
	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N
<i>Series I</i>								
5	—	—1.42	—0.35	—1.16	...	...	+ 0.08	+ 0.49
6	—0.54	—0.41	—0.08	—0.40	...	...	+ 0.29	+ 1.07
7	—	—0.78	—0.36	—0.11	...	...	0.00	+ 0.09
8	—0.62	—0.75	—0.53	—0.38	...	...	—0.40	—0.12
9	—0.75	—0.83	—0.46	...	...	...	+ 0.05 <sup>1</sup>	+ 0.45
<i>Series II</i>								
10 <sup>2</sup>	—0.23	...	...	—0.04	+ 0.03	...	...	+ 0.56
11	—0.71	...	...	—0.24	—0.04	...	...	+ 0.65
12	—0.88	...	...	—0.87	—0.05	...	...	+ 0.01
<i>Series III</i>								
15	...	...	...	...	—0.79 <sup>3</sup>	+ 0.07 <sup>3</sup>	...	+ 0.34
16	...	...	...	...	—0.26 <sup>3</sup>	0.00 <sup>3</sup>	...	+ 0.43
17	...	...	...	...	+ 0.54 <sup>3</sup>	...	...	+ 1.27
18	...	...	...	...	+ 0.59 <sup>3</sup>	...	...	+ 0.71
19	...	...	...	...	—0.19 <sup>3</sup>	...	...	+ 0.14
20	...	...	...	...	+ 0.16 <sup>3</sup>	...	...	+ 0.27
Mean	—0.62	—0.84	—0.36	—0.46	0.00	+ 0.04	0.00	+ 0.45

<sup>1</sup> Studied at a lysine level 0.56 gm per day.

<sup>2</sup> Same as subject 6.

<sup>3</sup> The daily methionine intake was 0.29 gm.

## INDICAN EXCRETION BY RATS FED RAW SOYBEAN OIL MEAL

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### INTRODUCTION

Although there have been occasional publications concerning indican (potassium 3-indoxyl sulfate), the attitude toward research on this subject has been aptly described by Meiklejohn and Cohen ('42): "For nearly a quarter of a century, there has been a general absence of interest in the significance of the urinary excretion of indole derivatives. The doctrine established by tradition that urinary indoles are derived from putrefactive processes in the intestines apparently has made further investigation of this subject unprofitable." The authors are aware of no direct proof that indican arises from intestinal putrefaction or more particularly from tryptophan degradation although such may be presumed from available data. Underhill and Simpson ('20) reported that indican was increased by a meat diet and that only a trace of indican was excreted on a gelatin diet. On the other hand, Sherwin and Hawk ('14) reported that indican excretion continued in a dog which was fasted for 117 days. The *in vivo* formation of indoxyl by means other than putrefaction and from compounds other than indole or its immediate derivatives has

<sup>1</sup> Published with the approval of the Director as Paper no. 716, Journal Series, Nebraska Agricultural Experiment Station. Some of these data were taken from a thesis presented by Djanhanguir Mohammad-Abadi to the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the M. S. Degree, June 13, 1955. A preliminary report was given at the annual meeting of the American Institute of Nutrition, San Francisco, April 11-15, 1955.

selected diets of women furnished 1.7 to 8.6 gm of lysine daily (Futrell et al., '52; Reynolds, Futrell and Baumann, '53). Moreover Block and Bolling ('51) have calculated that the "average" American diet supplies 5.2 gm of lysine per day, and that even the diet of the lowest income urban group yields 4.0 gm of lysine.

*DL-Valine.* With all other factors constant for a given subject the use of the racemic mixture as the source of L-valine in series III (table 6) had little effect on the nitrogen balances of these subjects. The averages of the mean daily

TABLE 6

*Mean daily nitrogen balances on 1.46 gm of L-valine and on 2.92 gm of DL-valine in the amino acid supplements*

SUBJECT	MEAN DAILY NITROGEN BALANCE <sup>1</sup>	
	1.46 gm L-Valine	2.92 gm DL-Valine
	gm	gm
15	+ 0.07	+ 0.16
17	+ 0.54	— 0.29
18	+ 0.59	+ 0.06
19	— 0.19	— 0.26
20	+ 0.16	+ 0.31
Mean	+ 0.23	0.00

<sup>1</sup> All subjects received 0.29 gm of methionine and about 0.5 gm of cystine per day. Subject 15 received 0.50 gm of lysine per day, while the remaining subjects received 0.40 gm per day.

nitrogen balances for all subjects were + 0.23 gm (— 0.19 to + 0.54) on the L-valine and 0.00 gm (+ 0.29 to + 0.31) on the DL-valine.

#### SUMMARY

The effects of various levels of lysine intake on nitrogen balance were studied in 14 women maintained on a semi-synthetic diet in which about 95% of the total nitrogen was furnished by pure amino acids and diammonium citrate. From data obtained in this experiment, it appears that 0.40 to 0.50 gm lysine per day is adequate for the establishment of nitrogen balance in women under these conditions.

nine,<sup>2</sup> vitamins (including 2  $\mu$ g vitamin B<sub>12</sub>), minerals, starch and fat (for details, see Borchers and Ackerson, '51). Urine samples, collected under mineral oil, were obtained on alternate two-day periods beginning on the 4th day of feeding. Indican was determined by a quantitative modification of the Obermayer test as described in detail by Zacherl ('33). Results are expressed as milligrams of indican excretion/day/100 gm body weight. Because of wide variations in excretion by the same animal in successive periods, data for progressive changes with time in indican excretion are not presented.

#### EXPERIMENTS AND RESULTS

Rats fed raw soybean oil meal gained 2.93 gm/day and excreted 3.83 mg of indican/100 gm body weight/day; those fed autoclaved soybean oil meal gained 4.15 gm and excreted 0.89 mg of indican. These results are itemized in table 1, experiment 1.

In the second experiment, 5% of crude trypsin powder was added to each ration. A previous report (Borchers and Ackerson, '51) established that the addition of 5% of crude trypsin equalized the growth rate of rats fed autoclaved versus raw soybean oil meal (addition of 5% of casein was ineffective). In experiment 2, growth was similar for autoclaved and raw soybean oil meal plus trypsin. However, indican excretion on the raw soybean ration plus trypsin continued at a high level as in experiment 1.

Wellers ('53) reported that dietary indole did not depress growth provided adequate cystine or methionine was included in the ration. His publication gave no data on indican excretion. Hence, it was necessary to establish the effect of indole on rats fed soybean rations and to determine the actual level of indican excretion after indole feeding. In experiment 3, indole was fed at levels of 0.05 and 0.1% in an autoclaved soybean ration. These levels did not affect the growth rate. Indican excretion on 0.05% of indole was approximately equal

<sup>2</sup>DL-Methionine, courtesy of The Dow Chemical Company, Midland, Michigan.

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to, and on 0.1% of indole twice that of rats fed raw soybean oil meal in experiments 1 and 2. Similar results were obtained when 0.1% of indole was added to a raw soybean oil meal ration. The average amount of indican excreted at either the 0.05 or 0.1% level accounted for approximately 40% of the indole intake. Whether the remainder was not absorbed or was metabolized in some other manner was not determined in these experiments. Feeding of indole above 0.1% has resulted in some depression of the growth rate which was due, in part, to reduced food consumption.

If indican arises from bacterial action in the intestine, suppression of bacterial growth should reduce indole production and indican excretion. Furthermore, if indican or its precursors or bacterial action in general are the cause of growth depression, suppression of intestinal bacteria should not only decrease indican excretion but should as well increase the growth rate of rats fed raw soybean oil meal. Since earlier experiments in our laboratory with antibiotics fed at moderate levels had shown no effects on growth of rats fed autoclaved or raw soybean oil meal rations, streptomycin sulfate was fed at a level of 0.1%. In this experiment, the usual difference in growth rate between animals fed autoclaved and raw soybean oil meal was observed. However, indican excretion was reduced for both the autoclaved and raw soybean rations; the excretion was approximately equal for the two rations as shown in experiment 4 of table 1. The 0.1% level of streptomycin has, in some experiments, stimulated the growth rate of rats fed raw soybean oil meal. A more detailed report of these feedings will be published later.

#### DISCUSSION

These investigations were conducted to determine whether a causal relationship existed between the increased excretion of indican and the reduced growth rate of rats when raw soybean oil meal was fed. If the assumption is made that possible intestinal putrefactive compounds, such as indole,



and rubidium did not gain so well as those receiving rubidium alone, indicating that when potassium was present rubidium acted as a poison. He observed nervous conditions in rubidium-fed rats. Certain histological changes were found in rats receiving potassium-deficient diets and also in those receiving diets containing both rubidium and potassium, but not in those receiving rubidium alone.

The biological effects of rubidium assume an added interest since radioactive rubidium now is used as a tracer for potassium (Love, Romney and Burch, '54; Threefoot, Ray and Burch, '55; Burch, Threefoot and Ray, '55). Love and Burch ('53), who used  $\text{Rb}^{86}$  as a tracer in an *in vitro* study of erythrocyte electrolyte metabolism, pointed out that absolute reliance on the metabolic similarity of rubidium and potassium was not justified.

In view of the limited information on the effects of ingested rubidium and of the somewhat conflicting findings on rubidium when used with or in place of potassium, this study of feeding different levels of rubidium in various combinations with potassium and sodium in a purified diet was undertaken.

#### EXPERIMENTAL

*Experiment 1.* The first experiment was on the effects of feeding different levels of rubidium in the purified diet with and without sodium. The 13 experimental groups used in this study consisted of random selections of two male and two female weanling rats, 21 to 27 days of age, weighing between 33 and 63 gm. The rats were individually housed in cages constructed with open-mesh floors which allowed droppings to pass through. The animal room was kept at a temperature of 75 to 80°C.

The synthetic basal diet used was patterned after those of Sporn et al. ('47) and Meyer et al. ('50). Alterations were made in the proportions of the alkali metals as shown in table 1.<sup>5</sup> This basal diet proved to be reasonably adequate

<sup>5</sup> Analysis of the basal diet revealed it was not so free of sodium as expected. It contained 0.006% of sodium, mostly from the casein used.

marked decrease in indican excretion, was without effect on the comparative growth rates of rats fed autoclaved and raw soybean oil meal. Since the reduced growth rate with raw soybeans persisted while indican excretion was markedly reduced, it seems unlikely that events leading to the excretion of indican have any effects on the growth rate. Or, the factors which reduce the growth rate of rats fed raw soybean oil meal are operating in the absence of indican excretion. Therefore, the reduced growth rate and increased indican excretion following raw soybean oil meal feeding are unrelated phenomena; neither may be regarded as a cause or a result of the other.

The question as to the actual source or cause of the increased indican excretion remains unanswered. The assumption that bacterial putrefaction is a factor seems warranted in view of the suppression of indican excretion after streptomycin feeding. In this connection, Carroll et al. ('52) have concluded, on the basis of chromic oxide marker experiments, that a greater proportion of the nitrogen reached the cecum when rats were fed raw soybean oil meal than when fed autoclaved meal. These authors then reasoned that much of this cecal nitrogen must be absorbed from the cecum, presumably as putrefactive products. Such cecal absorption would then account for the generally observed similar digestibility values for raw and autoclaved soybean oil meal. However, similar marker studies in our laboratory (Borchers, '53) failed to substantiate the basic observations of Carroll et al. ('52), thus vitiating their reasoning that cecal absorption was an important factor in raw versus autoclaved soybean digestibility studies.

#### SUMMARY

Rats fed raw soybean oil meal were found to excrete about 4 times as much indican and to grow at about three-fourths the rate of rats fed autoclaved meal. Addition of crude trypsin powder to the rations equalized the growth rate without affecting indican excretion. Addition of indole to rations

TABLE 3

Sodium, potassium and rubidium contents of diets; average weights, and survival times of rats

## Experiment 1

DIET <sup>1</sup>	CONTENTS IN DIETS				NO. RATS	AVERAGE WEIGHT						SURVIVAL TIME		
	Na	K	Rb	%		0 days	10 days	20 days	40 days	80 days	120 days	Min.	Max.	Mean
	%	%	%	%		gm	gm	gm	gm	gm	gm	days	days	days
A	0.39 (0.36) <sup>2</sup>	0.60 (0.76)	...	(0.004)	4	55	102	138	200	268	288	180	300+	101
B	0.00 (0.006)	0.29 (0.28)	0.00 (0.000)	0.00 (0.000)	4	47	63	71	92	139	202	262	300+	74
C	0.20 (0.20)	0.29 (0.32)	0.00 (0.000)	0.00 (0.000)	4	46	81	139	177	242	273	180	300+	37
D	0.00 (0.009)	0.29 (0.27)	0.01 (0.009)	0.01 (0.009)	4	48	65	76	106	152	196	141	300+	17
E	0.20 (0.19)	0.29 (0.28)	0.01 (0.011)	0.01 (0.011)	4	45	84	120	188	259	300	300+	300+	27
F	0.00 (0.008)	0.29 (0.29)	0.10 (0.14)	0.10 (0.14)	4	47	64	71	92	134	185	200	300+	12
G	0.20 (0.19)	0.29 (0.28)	0.10 (0.14)	0.10 (0.14)	4	50	79	118	175	218	239	176	300+	14
H	0.00 (0.010)	0.29 (0.29)	0.20 (0.21)	0.20 (0.21)	4	44	60	70	90	111 <sup>3</sup>	127 <sup>4</sup>	64	140	101
I	0.20 (0.19)	0.29 (0.28)	0.20 (0.21)	0.20 (0.21)	4	53	85	116	141 <sup>3</sup>	171 <sup>4</sup>	...	24	102	74
J	0.00 (0.008)	0.29 (0.30)	0.30 (0.30)	0.30 (0.30)	4	51	63	70	74 <sup>3</sup>	...	...	29	54	37
K	0.20 (0.18)	0.29 (0.32)	0.30 (0.30)	0.30 (0.30)	4	49	76	106 <sup>4</sup>	...	...	...	15	28	17
L	0.00 (0.007)	0.29 (0.30)	0.40 (0.37)	0.40 (0.37)	4	47	61	62	...	...	...	21	38	27
M	0.20 (0.15)	0.29 (0.34)	0.40 (0.40)	0.40 (0.40)	4	52	76	...	...	...	...	12	14	12

<sup>1</sup> Diet A was Purina Laboratory Chow. Diets B through M were composed of purified ingredients.<sup>2</sup> Number outside parentheses is calculated quantity added. Number within parentheses is content by analysis.<sup>3</sup> Only three surviving rats at this stage.<sup>4</sup> Only one surviving rat at this stage.<sup>5</sup> Only two surviving rats at this stage.

# EFFECT OF OVARECTOMY AND ADMINISTRATION OF OVARIAN HORMONES AND TESTOSTERONE ON NICOTINIC ACID METABOLISM OF RATS<sup>1,2</sup>

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In a previous report on the metabolism of nicotinic acid in pregnancy (Lojkin, Wertz and Dietz, '52), the theory was offered that the increased urinary excretions of metabolites of nicotinic acid observed in pregnant women and rats might possibly be attributed to the changes that occur in endocrine functions during pregnancy. Although the secretory activity of several hormones changes during pregnancy, it appeared of special interest to investigate the role of estrogen and progesterone, which are present in greatly increased concentrations during pregnancy, and of testosterone, which under certain conditions can inhibit (Turner, '55) or be inhibited (Martin et al., '55; Pincus and Dorfman, '55) by ovarian hormones.

A few short preliminary experiments were designed to observe how these hormones affect the urinary excretion of N'-methylnicotinamide (MNA) and the acid-hydrolyzable metabolites of nicotinic acid (NA) by the female rat. These tests indicated that the administration of testosterone propionate produced an immediate response, which consisted of

<sup>1</sup> Contribution 1037 of the University of Massachusetts Agricultural Experiment Station.

<sup>2</sup> Read by title before the American Institute of Nutrition, Federation of American Societies for Experimental Biology Meeting, Atlantic City, 1956.

and all 6 females lived more than 300 days. All mothers killed their first litters but 11 of 30 young of the second litters survived. Five second generation females had young resulting from the first mating, but only one litter was raised. In a second mating, three of 4 females gave birth to young, but only one litter was raised. Two females had a third litter, but no young were raised. One 4th generation litter was born to a female in this dietary group.

*Experiment 2.* A second experiment was conducted using a basal diet lower in content of alkali metals. In this study, diets contained different levels of sodium, potassium and rubidium, both alone and in combination. In experiment 1, no variation was made in potassium content.

The basal diet was the same as shown in table 1, with the following modifications. Vitamin-free casein was substituted for alcohol-extracted casein, since the former was found to contain less sodium and potassium. Potassium salts were not used in the mineral mix.  $\text{NH}_4\text{I}$  was substituted for  $\text{KI}$ . Calcium and phosphorus were supplied by 48.90% of  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ , and 31.76% of  $\text{CaCO}_3$  in the mineral mixture. Diets and tissues were analyzed for sodium, potassium and rubidium as in experiment 1.

In experiment 2 the groups of rats are designated by symbols indicating the kinds and amounts of alkali metals added to the basal diet (table 3). A single symbol indicates a content of 0.25% of that element and a double symbol twice that amount (e.g. Rb, 0.25% rubidium; NaK, 0.25% sodium and 0.25% potassium; KK, 0.50% potassium). B.O. designates basal diet only and L.C., laboratory chow (control group). Equimolar quantities of the alkali metals were not used since an amount of rubidium equimolar to desirable experimental levels of potassium and sodium could be tolerated but a few days.

In experiment 2 weanling rats from the stock colony, 24 to 28 days of age, were randomized into 12 groups, each consisting of 4 males and 4 females. The rats were placed in individual cages, kept in air conditioned space, and given

induced by ovariectomy and by injections of a combination of progesterone and estrone, and testosterone propionate.

#### METHODS AND PROCEDURES

Adult female albino rats, weighing approximately 300 gm, from a strain originally obtained from the Connecticut Agricultural Experiment Station, were used in this investigation. They were fed ad libitum a stock breeder's ration consisting of 10% meat scraps, 60% whole wheat, and 30% whole milk powder, to which 1.5% NaCl was added. This ration contained 44  $\mu$ g of nicotinic acid and 1.5 mg of tryptophan per gram. The animals were maintained in metabolism cages continuously throughout the experimental periods. Records of the daily food intake were kept, and the weights of the rats were recorded at intervals during the study. Twenty-four-hour urine samples were collected under toluene, diluted to volume, filtered, and stored frozen at  $-20^{\circ}\text{C}$ . until analyzed. Determinations of MNA were made on each individual daily sample of urine by the fluorometric method of Huff and Perlzweig ('47). Representative samples were analyzed for nicotinic acid by the microbiological method of the Association of Vitamin Chemists ('51). Samples from the ovariectomized rats in experiment I were analyzed for tryptophan microbiologically with *Leuconostoc mesenteroides* as the test organism by the method of Steele et al. ('49).

*Experiment 1.* Six adult female rats were placed in individual metabolism cages and 24-hour urine collections made for from 5 to 12 days. At the end of this period, the rats were ovariectomized. In 4 to 6 weeks after the operation, the 6 ovariectomized rats as well as 6 intact rats were placed in individual metabolism cages and 24-hour urine collections made for a pre-injection period of at least 9 days, an injection period in which a combination of 4 mg of progesterone and 0.5  $\mu$ g of estrone in sesame oil was administered intramuscularly for 10 consecutive days, and a post-injection period of sufficient duration for the excretion of MNA to approach the pre-injection level. This dosage of progesterone and estrone

daily care. They were fed ad libitum, as in experiment 1, and a record of food consumption was kept. Live rats were weighed every 4 days, and those that died were weighed soon after death.

Average weights of each group of rats at certain periods and survival times are in table 3. Rats receiving basal diet only (B.O.) failed to grow, those receiving sodium (Na and NaNa) grew little; those receiving rubidium (Rb and RbRb) grew at early stages comparable to those receiving potassium (K and KK). The sodium-rubidium (NaRb) and potassium-rubidium (KRb) diets produced comparable early growth. Rats receiving the sodium-potassium combination (NaK) grew better than those of any other group, except for the controls (L.C.), which averaged a little heavier.

Rats eating a diet practically devoid of sodium, potassium and rubidium (B.O.) survived a mean of 53 days. Adding rubidium (Rb and RbRb) decreased mean survival time to 23 days and 13 days, respectively. Adding sodium (Na and NaNa) alone, decreased survival time to 37 and 33 days, respectively. Adding potassium (K and KK), however, increased survival time to 99 days or more. Survival time on the sodium-rubidium (NaRb) diet and the rubidium (Rb) diet was approximately the same. Rats receiving the potassium-rubidium combination (KRb) survived longer than those receiving rubidium (Rb) alone but died earlier than those receiving potassium (K) alone.

When an animal died, it was dissected and gross examination made of internal organs. Symptoms of rubidium toxicity observed before death of the rats were the same as described in experiment 1. Post mortem findings were not conclusive as to the cause of death. Animals receiving no alkali metal except sodium were bloated before death, and post mortem examination revealed abnormal quantities of a free, watery fluid in the abdominal and thoracic cavities.

After death of each animal the following organs were removed, weighed and preserved for analysis: lungs, heart, liver, kidney, brain. In addition, samples of bone and muscle

also for individual rats of the same strain, have been previously reported (Perlzweig et al., '43; Lojkin et al., '52).

There were also quantitative variations in the responses of individual animals to both ovariectomy and hormonal therapy. However, the trend in the changes of the excretion of the metabolites of nicotinic acid induced by these treatments was similar in all the animals studied. Ovariectomy

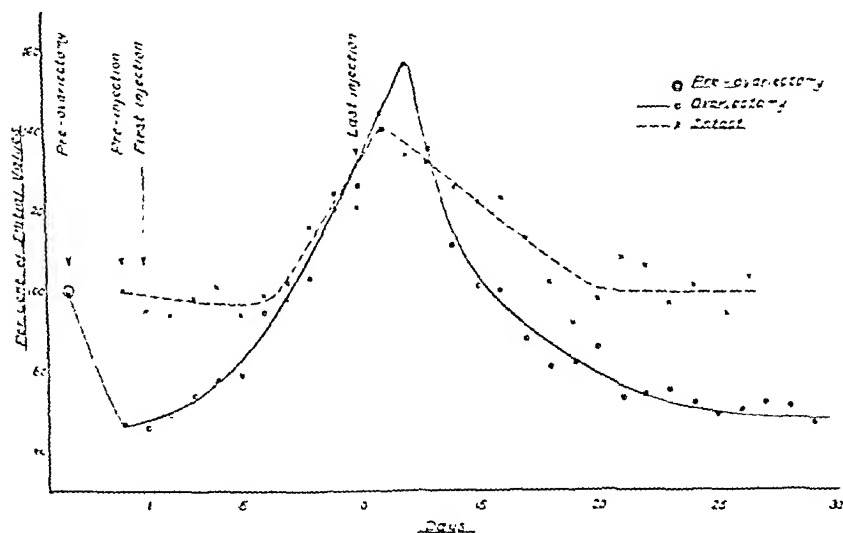


Fig. 1 Average daily urinary excretions of N'-methylnicotinamide for intact rats and ovariectomized rats injected with a combination of 4 mg of progesterone and 0.5  $\mu$ g of estrone for 10 consecutive days, expressed as percentages of initial values. (The average initial value for the intact rats is 196  $\mu$ g/24-hour; the average initial pre-ovariectomy value is 226  $\mu$ g/24-hour.)

resulted in a marked drop in the MNA and NA output of all the rats. The decreases averaged 33 and 23% of the initial values for MNA and NA, respectively, and were statistically significant at the 1% probability level. The excretion of the metabolites of nicotinic acid by the ovariectomized rats remained unaltered during the first two days of hormonal administration. A gradual augmentation was noticeable in most of the rats on the third day (fig. 1). The MNA and NA excretion approached the initial pre-operative levels on the 7th



TABLE 4

*Average sodium, potassium and rubidium contents of various tissues and organs of rats fed diets containing different amounts of alkali metal cations in experiment 2*

DIETARY GROUP <sup>1</sup>	APPROXIMATE DIETARY CONTENT			CONTENT, DRY BASIS						
	Na	K	Rb	Lungs	Heart	Liver	Kidneys	Muscle	Brain	Bone
	%	%	%	%	%	%	%	%	%	%
<i>Sodium</i>										
B.O.	0.0	0.0	0.0	0.19	0.32	0.15	0.26	0.24	0.37	0.35
Na	0.25	0.0	0.0	0.73	0.63	0.55	0.77	0.91	0.57	0.57
K	0.0	0.25	0.0	0.25	0.21	0.14	0.21	0.15	0.33	0.26
Rb	0.0	0.0	0.25	0.38	0.43	0.27	0.44	0.24	0.50	0.46
NaNa	0.50	0.0	0.0	0.79	0.70	0.64	0.76	0.66	0.51	0.53
NaK	0.25	0.25	0.0	0.39	0.26	0.19	0.42	0.12	0.37	0.43
NaRb	0.25	0.0	0.25	0.84	0.59	0.13	0.57	0.38	0.56	0.49
KK	0.0	0.50	0.0	0.13	0.09	0.06	0.16	0.04	0.13	0.24
KRb	0.0	0.25	0.25	0.24	0.27	0.16	0.28	0.23	0.40	0.34
RbRb	0.0	0.0	0.50	0.41	0.63	0.24	0.33	0.17	0.40	0.32
L.C.	0.38	0.60	0.0	0.41	0.25	0.30	0.42	0.40	0.38	0.48
<i>Potassium</i>										
B.O.	0.0	0.0	0.0	0.75	0.82	0.77	0.85	0.80	1.02	0.28
Na	0.25	0.0	0.0	0.89	0.93	0.82	0.97	0.88	0.97	0.37
K	0.0	0.25	0.0	0.86	0.85	0.54	0.91	1.03	1.06	0.30
Rb	0.0	0.0	0.25	0.43	0.43	0.48	0.50	0.63	0.87	0.19
NaNa	0.50	0.0	0.0	0.72	0.70	0.78	0.84	0.81	1.05	0.39
NaK	0.25	0.25	0.0	0.91	0.97	0.86	0.93	1.20	1.43	0.38
NaRb	0.25	0.0	0.25	0.73	0.58	0.64	0.69	0.57	1.34	0.23
KK	0.0	0.50	0.0	0.93	1.03	0.97	0.90	1.06	1.17	0.29
KRb	0.0	0.25	0.25	0.85	0.89	0.90	0.91	0.88	1.05	0.55
RbRb	0.0	0.0	0.50	0.57	0.55	0.59	0.56	0.58	1.05	0.29
L.C.	0.38	0.60	0.0	1.03	1.06	0.97	0.85	1.26	1.43	0.43
<i>Rubidium</i>										
B.O.	0.0	0.0	0.0	0.03	0.03	0.04	0.03	0.00	0.03	0.00
Na	0.25	0.0	0.0	0.01	0.00	0.02	0.02	0.00	0.02	0.00
K	0.0	0.25	0.0	0.02	0.00	0.01	0.02	0.02	0.01	0.00
Rb	0.0	0.0	0.25	0.89	0.82	1.18	1.19	1.52	1.04	0.42
NaNa	0.50	0.0	0.0	0.03	0.03	0.06	0.05	0.04	0.05	0.00
NaK	0.25	0.25	0.0	0.01	0.00	...	...	...	0.00	0.00
NaRb	0.25	0.00	0.25	1.54	1.17	1.50	1.71	1.36	1.62	0.42
KK	0.0	0.50	0.0	0.04	0.00	0.03	0.03	0.04	0.02	0.00
KRb	0.0	0.25	0.25	1.08	1.24	1.32	1.34	1.29	1.23	0.74
RbRb	0.0	0.0	0.50	1.55	1.66	1.56	1.51	1.70	1.85	0.77
L.C.	0.38	0.60	0.00	0.92	0.62	0.62	0.91	0.92	0.91	0.00

<sup>1</sup> See text, page 548, for notes.

<sup>1</sup> See text, page 568, for rubidium designations.

<sup>2</sup> Measurable but less than 0.01%.

and 5th day, respectively, and reached maximum values for MNA on the 11th or 12th day and for NA on the 9th to 12th day after the first injection. Declines in the excretion values started after the 11th to 15th day and continued for periods which varied in duration for the individual animals. On the average, the values returned to levels approaching the pre-injection values by the 25th day.

Administration of 4 mg of progesterone and 0.5  $\mu$ g of estrone to intact rats (fig. 1) induced changes in MNA and NA excretion which followed the general trend observed in ovariectomized rats, although a longer period of time was required for the occurrence of the response. On the average, the MNA and NA values started to rise after the 8th and 6th day, respectively, of hormonal administration. Although the results from the individual rats tended to fluctuate rather than to increase steadily from day to day, the mean values changed steadily from one period to another (table 1). The daily excretion values reached their maximum levels for the different rats after intervals that varied in duration from 11 to 15 days for MNA and from 8 to 12 days for NA. The excretions approached the pre-injection levels during the 20-to-25-day period.

Results of a comparison of the excretion values during the different periods of the experiment (table 1) indicated that the maximum MNA excretion of the ovariectomized injected rats exceeded the pre-injection values by 203  $\mu$ g or 134% and the pre-ovariectomy values by 129  $\mu$ g or 57%. The average for the 5-day period (11th to 15th day), in which the maximum response occurred, exceeded the pre-injection levels by 141  $\mu$ g or 93% and the pre-ovariectomy levels by 67  $\mu$ g or 30%. In the intact rats the maximum MNA values and the average values for the 5-day period during which the maximum MNA excretion occurred exceeded the average initial pre-injection values by 119  $\mu$ g or 61% and 63  $\mu$ g or 32%, respectively. All these differences were statistically significant.

A highly significant correlation between the MNA and NA excretion was found in each of the rats. In both the ovari-

TABLE 5

*Weight gains and balance data on rats during a 14-day trial<sup>1</sup>*

DIET <sup>2</sup>	CONTENTS IN DIET <sup>3</sup>			WT. GAIN 14 DAYS	FEED EATEN	WATER INTAKE	FECES (Dry wt.)	URINE (Est.) <sup>4</sup>
	Na	K	Rb					
	%	%	%	gm	gm	ml	gm	ml
LC	.36	.76	.004	88	195	331	65.2	68
BO	.001	.003	.000	1	81	321	4.07	130
Rb	.001	.001	.24	13	97	232	4.81	64
NaRb	.23	.013	.25	33	119	145	5.16	18
KRb	.001	.24	.26	21	121	271	5.82	42
NaKRb	.25	.25	.27	44	127	197	5.43	35

DIET	QUANTITY MINERAL INGESTED	EXCRETED IN URINE	EXCRETED IN FECES	TOTAL EXCRETED	TOTAL RETAINED
	mg	mg	mg	mg	mg

*Sodium*

LC	700	370	70	440	260
BO	0.5	* <sup>5</sup>	*	0.00	0.5
Rb	0.5	*	*	0.00	0.5
NaRb	273	90	5	95	178
KRb	1.3	*	*	0.00	1.3
NaKRb	318	115	4	119	198

*Potassium*

LC	1479	117	186	303	1176
BO	2.6	0	2	2	.6
Rb	9.3	15	2	17	(-7.7)
NaRb	15.4	14	4	18	(-2.6)
KRb	290	37	8	45	245
NaKRb	313	39	9	48	265

*Rubidium*

LC	7.8	*	*	0.00	7.8
BO	0.0	*	*	0.00	0.0
Rb	228	13	3	16	212
NaRb	296	14	5	19	277
KRb	314	68	8	76	238
NaKRb	343	63	5	68	275

<sup>1</sup> All data are averages from 2 rats.<sup>2</sup> Diets designated in same manner as in experiment 2.<sup>3</sup> Contents by analysis of diets.<sup>4</sup> Some volume lost by evaporation.<sup>5</sup> Not detected in analysis.

of the two groups of rats varied from 11 to 15 gm for the different periods, but indicated no consistent trend from period to period. Although the initial weights of the two groups of rats were the same, the average initial excretion values for MNA and NA were somewhat higher for the group of rats that were to be ovariectomized than for the group of normal intact rats. These experiments did not indicate any significant correlations between the metabolites of nicotinic acid excreted and the weights, or food intakes, of the rats.

TABLE 2

*Average urinary excretion of N'-methylnicotinamide (MNA) and the acid-hydrolyzable metabolites of nicotinic acid (NA) by ovariectomized and intact rats injected with 8 mg testosterone propionate for 5 consecutive days, expressed as percentages of initial values*

NO. OF RATS Period	Days	OVARIECTOMIZED RATS			INTACT RATS		
		3 MNA	3 NA	3 Food intake	2 MNA	2 NA	2 Food intake
		$\mu\text{g}/24 \text{ hr.}$	$\text{gm}/\text{day}$		$\mu\text{g}/24 \text{ hr.}$	$\text{gm}/\text{day}$	
		Initial values			Initial values		
Pre-injection		140	78	13	161	97	14
		% of initial values <sup>1</sup>			% of initial values <sup>1</sup>		
Pre-injection		100	100	100	100	100	100
Injection	1-5	57	74	104	50	78	109
Post-injection	6-8	83	104	110	49	89	100
	9-11	110	117	104	61	96	104
	12-14	124	131	110	75	103	106
	15-18	118	148	110	110	121	112

<sup>1</sup> Averages of the individual percentages.

### *Effect of testosterone*

Data regarding urinary excretions of MNA and NA as well as the food intake of ovariectomized and intact rats injected with testosterone propionate are presented in table 2. The figures represent the averages of the results obtained from analyses of separate 24-hour samples for each rat for the pre-injection period, a 5-day injection period, and 3-day post-

longevity that precludes full metabolic interchangeability. Heppel and Schmidt ('38) expressed the same idea.

When a total of less than 400 mg of alkali metals was fed to rats during a 14-day balance study, rubidium resembled potassium more than sodium in that the major part was retained in the body. Ingested rubidium was similar to sodium and potassium in that the main path of elimination was by the kidneys rather than by the digestive tract. This is in accord with findings of Mendel and Closson ('06) and Freedberg, Pinto and Zipser ('52).

Purified diets that contained as little as 0.1% of rubidium in presence of 0.25% of potassium were toxic, as judged by growth and reproductive performance. Toxicity increased as the rubidium content of the diets was increased, and 0.20% of rubidium markedly decreased survival time. Toxicity also was increased by the addition of sodium to the diets, although growth up to a short time before death was improved by sodium. Diets containing 0.25% of rubidium were more toxic in the absence of potassium than those that also contained 0.25% of potassium.

The more toxic effects of dietary rubidium in the absence of potassium, or presence of only small amounts, is in agreement with the findings of Mitchell et al. ('21) and Heppel and Schmidt ('38). The present observations that rats lived longer on a diet containing both potassium and rubidium than on one containing rubidium alone is not in agreement with the report by Follis ('43). However, it is difficult to compare present results with those of other workers cited because of the differences in experimental conditions.

Heppel and Schmidt ('38) analyzed carcasses of rats fed two levels of rubidium and potassium and reported that in both groups there were differences in molar concentrations of rubidium and of potassium but that the sum of these two elements was about the same in both groups. One might expect a similar finding in this study if the sums of molar contents of rubidium plus potassium in the tissues were calculated. All tissues from rats eating diets containing rubidium

activity induced by ovariectomy resulted in marked decreases in the excretion of both MNA and NA. The initial pre-ovariectomy MNA and NA excretion levels exceeded those of the ovariectomized rats by approximately 50 and 30%, respectively.

Increases in the amount of ovarian hormones present in the animal's body as the result of the administration of a combination of 4 mg of progesterone and 0.5  $\mu$ g of estrone, induced definite increases in the output of nicotinic acid metabolites. In the rats whose MNA and NA excretion had been lowered by ovariectomy the total rise and the rate of augmentation of the excretion of these metabolites were especially pronounced.

The increase in the MNA and NA excretion of the ovariectomized animals became noticeable on the third day after the beginning of the hormonal administration. The MNA and NA values of these rats increased by approximately 50 and 30%, respectively, and thus reached the levels of their initial pre-operative excretion values, before the excretion of the intact rats began to rise. Subsequent elevations in the excretion values of the nicotinic acid metabolites proceeded at approximately equal rates in both groups of rats. The maximum MNA and NA excretion values reached by the ovariectomized rats exceeded their initial pre-ovariectomy levels of excretion by 57 and 48%, respectively, and those of the intact animals exceeded their initial MNA and NA values by 61 and 46%, respectively. These results indicate that under the conditions of these experiments, hormonal therapy could elevate the excretion of nicotinic acid metabolites to a certain definite per cent above the initial values, regardless of whether the hormones were administered to ovariectomized rats, which had low pre-injection excretion values, or to intact animals, which had higher pre-injection MNA and NA excretion levels.

In the study of the metabolism of nicotinic acid in pregnancy (Lojkin et al., '52), it was observed that urinary excretion of MNA and NA in pregnant rats increased gradually with the duration of pregnancy, and exceeded, during the

tissues. There were small reductions of the molar concentrations of the sum of sodium plus potassium in the tissues of rats eating diets containing rubidium, but the sum of molar concentrations of alkali ions was increased markedly. Rubidium replaced only a small amount of the sodium and potassium of tissues and apparently was retained in addition to them.

The inclusion of sodium in diets containing rubidium increased early growth of rats but decreased survival time. The presence of potassium in diets containing rubidium caused better growth of rats and longer survival than rubidium alone. Rubidium appeared to substitute only partially for potassium.

In blood, rubidium was found in much higher concentrations in the cells than in the serum fraction. The kidney was the main path for elimination of rubidium.

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tories, the decreases in the excretion of nicotinic acid metabolites resulting from injections of testosterone propionate, were of the same order of magnitude for the ovariectomized rats and for their intact controls. This similarity would indicate that the decreases could not be attributed, at least entirely, to inhibiting effects between the ovarian hormones and testosterone. However, the prolonged depression in the levels of MNA and NA excretion in the intact rats, compared to the very rapid return to higher levels of excretion in the ovariectomized rats, suggests that the presence of ovarian hormones may to a certain degree influence in some way, possibly indirectly, the effect of testosterone on the metabolism of nicotinic acid.

#### SUMMARY

Hormonal activity of female rats was changed by means of ovariectomy and by administration of ovarian hormones and of testosterone propionate.

In a group of rats that excreted on the average 225  $\mu$ g/24-hour of N'-methylnicotinamide (MNA) and 133  $\mu$ g/24-hour of the acid-hydrolyzable metabolites of nicotinic acid (NA), ovariectomy resulted in a mean decrease of 33 and 23%, respectively, in the urinary excretion of MNA and NA.

Ten daily injections of a combination of 4 mg of progesterone and 0.5  $\mu$ g of estrone resulted in statistically significant rises in the excretion of the nicotinic acid metabolites. The maximum MNA and NA excretion by the ovariectomized rats exceeded their pre-injection excretion values, on the average, by 134 and 93%, respectively. The percentages of increases of the excretions of ovariectomized and intact animals over the levels of the initial excretions were of the same order of magnitude, regardless of whether the hormones were administered to the intact or the ovariectomized animals. The maximum rises averaged approximately 59 and 47%, respectively, for MNA and NA excretions.





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no attempt to determine the accuracy of single urine samples as estimates of the true excretion rate. The advantages or disadvantages of the three parameters—time, creatinine, and urine volume—to which the excretion may be compared are not known in quantitative terms. We have attempted to make such estimates in the present paper with regard to riboflavin excretion. Urine was collected every two or 4 hours from a series of subjects and the variation in riboflavin excretion per hour, per milligram of creatinine, and per milliliter of urine has been compared.

Regardless of the method of expressing the results, it may be assumed that the longer the collection period, the more accurately the sample will estimate the true average rate of excretion. From the data obtained, it was also possible to estimate the improvement in accuracy obtainable by increasing the time of urine collection.

#### EXPERIMENTAL

Data were obtained in two separate experiments. In the first, the subjects were 4 mentally-subnormal men between the ages of 20 and 43 years. Two had mongolism and the other two were mentally subnormal following brain damage. We have been unable to show a significant difference in the excretion of riboflavin in mongoloids as compared to other types of mentally deficient patients. The subjects in this study consumed their usual diets in the same institutional dining room and records were kept of the kind and weight of food consumed. Estimates of the riboflavin intake were made using standard food tables. The diets contained considerable amounts of milk and were accordingly high in riboflavin. The usual intakes were from 2.5 to 3.5 mg of riboflavin per day and on the two days during which urine collections were made the mean intakes were between 3.2 and 3.4 mg per day. Meal hours were uniform with breakfast at 7 A.M., lunch at 12:30, and supper at 5 P.M. The three meals provided approximately 25, 45, and 30% of the total riboflavin

# THE INFLUENCE OF CHLORTETRACYCLINE AND VITAMIN B<sub>12</sub>, ALONE AND IN COMBINATION, ON NITROGEN UTILIZATION BY GROWING SWINE<sup>1</sup>

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Numerous attempts have been made to define the mechanism by which antibiotics exert their growth-promoting effects in swine since it was discovered (Jukes et al., '50) that an antibiotic, aureomycin, was the cause of greatly increased gains of growing swine fed a crude "Animal Protein Factor" supplement. Control of disease level (Speer et al., '50), sparing of niacin (Powick et al., '50), sparing of methionine (Cunha et al., '49), and sparing of crude protein (Cunha et al., '50; Catron et al., '52; Burnside et al., '54) have all been proposed as explanations for the mode of action of antibiotics. Diets adequate in the B vitamins and containing 15 or 18% of crude protein were of equal value for weanling pigs in the presence or absence of supplemental oxytetracycline (terramycin), but a possible sparing effect of oxytetracycline upon protein requirement was not indicated (Hofer et al., '52). It has been pointed out that for an antibiotic to exert a true sparing action on the protein requirement of pigs (Jensen et al., '55), it would be necessary for pigs fed low protein diets

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deviations. Other reasons for the use of logarithms were found in the observation that the distribution of the data tended to be skewed toward high values, and that the coefficient of variation for different subjects was of similar magnitude although the mean excretion rate varied. The values for the points, two standard deviations of either side of the geometric mean, were obtained and the antilogarithms of these values expressed as percentage of the mean value. Approximately 95% of the values are expected to fall within  $\pm 2$  standard deviations of the mean value.

Logarithms were not used for calculation of the standard deviations of the creatinine output.

We also desired to compare the relative variation in two-hour collections as compared to 4-hour collections and longer periods. For this purpose the riboflavin and creatinine excretions and urine volumes for each pair of two-hour periods were added together to give 4-hour periods, etc., and the excretion per milligram of creatinine and per milliliter of urine recalculated. Logarithms of the values were used for the statistical calculations as described above, and the results expressed in the same way. It is realized that these are not independent estimates but the findings are of practical interest.

Finally, in the subjects who consumed the low-riboflavin diet, the urinary excretion of riboflavin gradually declined during the collection period. In order to obtain a better estimate of the variation due to unknown causes, the deviations from the mean which could be accounted for by linear regression (due to the gradual decline in excretion) were subtracted. It is realized that the regression may not be linear but more extensive manipulation of the limited data available is probably not justified. Also, the expression of the variation about the line of regression as percentage of the mean excretion may be criticized. These factors may explain the somewhat greater variation seen in the data from the subjects consuming low riboflavin diets than in the subjects in the first experiment.

diets and environment, adjustment periods of at least 8 days between collections from the same pig, 7-day periods for collection of feces and 5-, 6- or 7-day periods for collection of urine. All collections were made using adjustable cylindrical swine metabolism cages patterned after those designed by Bell ('48).

The basal diet consisted of the following by weight: ground yellow corn, 82.8; solvent soybean oil meal, 9.8; meat scraps, 4.9; steamed bone meal, 2.0 and iodized salt, 0.5. Adequate amounts of vitamins A and D, riboflavin, niacin, pantothenic acid and choline were included in the diet along with a trace element mixture. This diet contained 14.1% of crude protein based on nitrogen determinations of the basic ingredients. The basal diet was fed unsupplemented (treatment 1) and with the following levels of supplements indicated per kilogram of diet: 22 mg of chlortetracycline (treatment 2), 11  $\mu$ g of vitamin B<sub>12</sub> (treatment 3), or a combination of the above levels of chlortetracycline and vitamin B<sub>12</sub> (treatment 4).

Each diet was fed at levels closely approximating 2.9, 3.5 and 4.1% of the live weight of the individual pigs during the different collection periods, thus making a total of 12 sub-treatments. The daily feed intake of each pig, based on the predicted mean weight for the pig while on collection, was held constant during the last 4 days of the adjustment period and all of the collection period.

All data have been summarized to present animal weights as mean weight expressed in kilograms for the time that the individual pigs were on collection. These mean weights of the individual pigs have been reduced to  $W_{\bar{x}}^{0.734}$  to eliminate insofar as possible the influence of difference in body weight,  $W$ , between different pigs and different periods (Brody and Procter, '32). This approach was recently used in studying nitrogen metabolism of pigs fed varying concentrations of dietary protein (Armstrong and Mitchell, '55). Armstrong and Mitchell fed their animals at a level of 5% of body weight raised to the power 0.900, while all pigs used in these investigations were fed on the basis of actual body weight.

tained by lengthening the collection above 12 hours is relatively small. Since the standard error of a mean is  $\frac{s.t. dev.}{\sqrt{N}}$ , where  $N$  is the number of samples, the number of samples of any particular size required to give the degree of accuracy desired may be estimated from figure 1.<sup>2</sup>

Textbooks widely quote the original observations of Shaffer ('08-'09) to the effect that the excretion of creatinine from hour to hour is as constant as it is from day to day. A calculation using the values obtained by Shaffer upon subject M.S. where the urines were collected in periods of two to two and one-half hours show a coefficient of variation of only 5.6%. The data from subject P.A.S. are similar with a coefficient of variation of about 6%. The original data of Folin ('05) taken from table XI, p. 116 of Hunter ('28) show a coefficient of variation on the 24-hour samples of about 4%. Thus, Shaffer's conclusion appears entirely justified. On the other hand, various authors have failed to find such constant rates of excretion (Albanese and Wangerin, '44; Clark et al., '51; Addis et al., '51). The data from table 214, p. 708 and table 327, p. 1034 of Macy's publication ('46) obtained with the children Jimmy and Frank give coefficients of variation of the 24-hour samples of 8.2 and 17.9% respectively of the mean daily excretions. A part of the increased variability observed by us, by Macy and others might be partially explained by meat consumption which may influence creatinine excretion somewhat (Karambelkar et al., '52) or by less than complete urine collections. The latter presumably show up when rather low values are followed by high values or the reverse. As Shaffer ('08-'09) says "It is by no means an easy matter without some practice to empty the bladder

<sup>2</sup> It would be expected that the standard deviation of the 4-, 8- and 24-hour samples could be obtained by dividing the standard deviation of the two-hour samples by  $\sqrt{2}$ ,  $\sqrt{4}$ , and  $\sqrt{12}$ , respectively, since 2, 4, and 12 two-hour collections are combined to obtain these samples. Such calculations essentially duplicate the values of figure 1. Since the number of subjects was only 4 for the two-hour samples and 7 for the 4-hour samples, and the total number of observations at each period was not large, the experimentally determined values were used to construct the figure.

## NITROGEN METABOLISM IN GROWING SWINE

TABLE 1  
Average nitrogen metabolism data for the pigs on the individual treatments<sup>1</sup>

TREAT- MENT	LEVEL OF FEED IN TREAT. AS PER CENT OF BODY WEIGHT	MEAN BODY WEIGHT kg	AV. DAILY INCREASE kg	AIR DRY FEED CON- SUMED DAILY gm	NITROGEN METABOLISM PER DAY				NITROGEN METABOLISM PER DAY PER VEG			
					Intake gm	Feces gm	Urine gm	Balance gm	Absorbed gm <sup>2</sup>	Urine gm	Balance gm	0.734
1	2.8	39.9	0.43	1138	25.70	5.26	10.41	10.03	1.38	0.70	0.68	
	3.5	43.5	0.71	1520	31.31	7.38	12.55	14.38	1.68	0.80	0.88	
	4.0	47.7	0.61	1859	41.97	9.87	14.37	17.73	1.86	0.83	1.03	
2	2.9	41.6	0.61	1188	26.83	5.58	10.23	11.02	1.36	0.67	0.69	
	3.5	44.0	0.60	1512	34.80	6.19	14.79	13.82	1.75	0.91	0.84	
	4.0	43.6	0.73	1724	38.92	8.14	15.77	14.71	1.89	0.97	0.92	
3	2.9	39.5	0.40	1143	25.80	5.25	10.73	9.82	1.36	0.72	0.64	
	3.5	42.5	0.61	1514	34.18	7.57	14.62	11.99	1.66	0.91	0.75	
	4.1	42.6	0.61	1710	38.73	8.50	14.76	15.47	1.90	0.94	0.96	
4	2.9	43.7	0.47	1279	28.88	5.05	13.40	10.43	1.46	0.82	0.82	
	3.5	44.4	0.65	1575	35.56	6.48	15.05	14.03	1.76	0.94	1.08	
	4.2	45.7	0.85	1924	43.43	7.74	17.54	18.15	2.12	1.04	1.04	

<sup>1</sup> Each figure represents the average of 6 collections.<sup>2</sup> Apparently absorbed nitrogen.<sup>3</sup> Average data for only the 5 pigs on which total data were obtained.



or not is immaterial for the present purposes since the samples were obtained from unpracticed persons and greater accuracy would not be expected in the field. Thus, a measure of the creatinine excretion does not appear to provide a

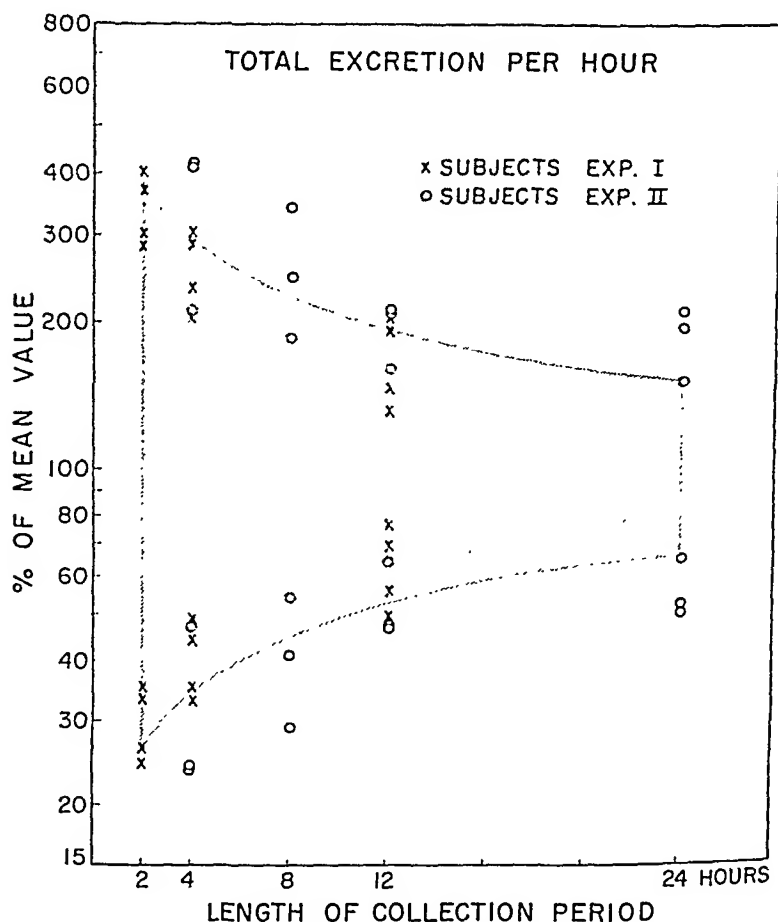


Fig. 3 The effect of the length of the collection period on the constancy of riboflavin excretion expressed on an hourly basis.

particularly accurate estimate of the time during which the urine was collected. Whether it is a suitable parameter for estimating the nutritional status, however, depends upon whether or not other baselines provide more accurate esti-

protein diet used in this trial may not have been sufficiently limiting in total nitrogen, particularly for the heavier pigs, to produce results which would indicate increased nitrogen retention due to chlortetracycline, vitamin B<sub>12</sub>, or a combination of the two. The level of feed intake used by these investigators is not known. The study reported here was intentionally conducted with a diet containing 14.1% of crude protein, a level of protein believed to be adequate but considerably below that recommended by the National Research Council ('53).

#### SUMMARY AND CONCLUSIONS

Growing barrow pigs weighing from 19.50 to 77.44 kg (mean 43.50 kg) and with metabolic size values of 8.49 to 23.35 (mean 15.64) did not show a significant increase in nitrogen retention when a 14.1% crude protein diet was supplemented with either 22 mg of chlortetracycline, 11 µg of vitamin B<sub>12</sub>, or a combination of the two, per kilogram of diet.

Highly significant correlations were found between body weight in kilograms raised to the power 0.734 and nitrogen retention within each of the three levels of feed intake used. Highly significant differences in nitrogen retention were demonstrated to exist due to level of feed intake.

Pigs consuming more total feed were able to utilize their nitrogen more efficiently for body gains as evidenced by the highly significant increases in nitrogen retention due to increased level of feed intake.

Neither chlortetracycline nor vitamin B<sub>12</sub>, nor a combination of the two, exerted a sparing action *per se* upon the protein requirement of growing swine fed the 14.1% protein diet used in this study.

#### ACKNOWLEDGMENTS

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It may be seen from these figures that single samples provide a very poor estimate of the actual rate of excretion regardless of whether the results are expressed on a time, creatinine, or urine volume basis. When the riboflavin ex-

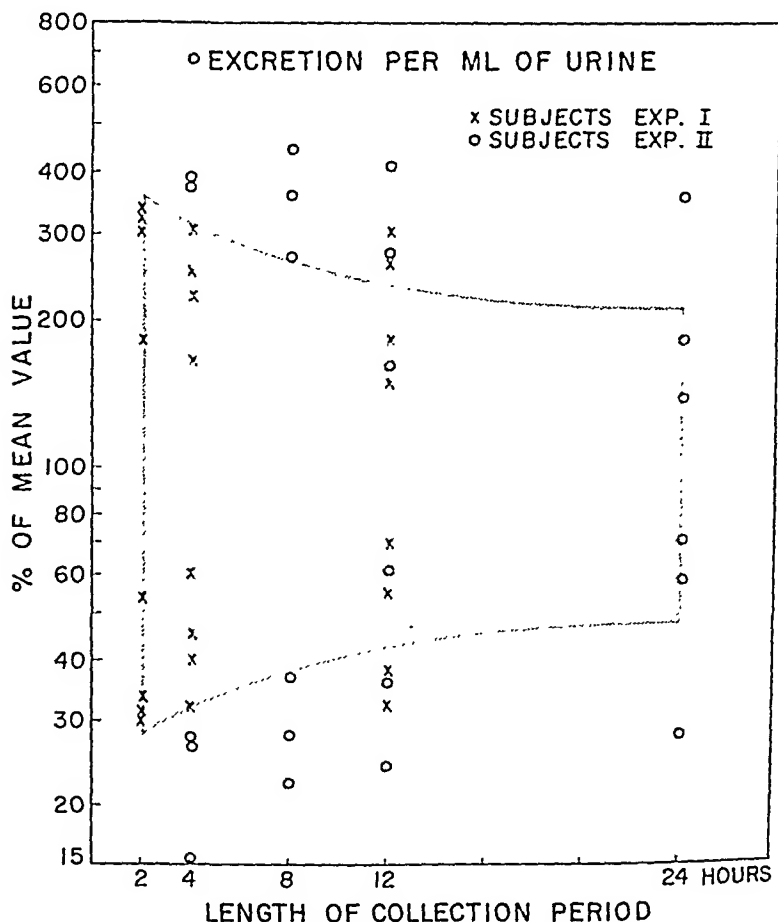


Fig. 4 The effect of the length of the collection period on the constancy of riboflavin excretion per milliliter of urine.

cretion is expressed per hour or per milligram of creatinine, the variation appears to be of the same order of magnitude. The difference between individuals appears to be much larger when the riboflavin is related to urine volume. Whereas some

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## DISCUSSION

In view of the great advantages of using randomly collected urine samples in field surveys, the results we have obtained are discouraging, but also thought provoking. From these data it appears that single urine samples provide a very poor estimate of the actual riboflavin excretion regardless of whether the excretion is related to time of collection, creatinine excretion or urine volume. Some satisfaction may be taken from the observation that riboflavin excretion per unit of creatinine is as constant, or perhaps more constant, than riboflavin excretion per hour. Thus, it will ordinarily be more satisfactory to measure creatinine than attempt to determine the time during which the urine is collected.

Since the accuracy of single samples improves as the collection periods become longer, emphasis should be placed upon getting a sample accumulated over the longest period possible. Also, it would appear that the influence of meals and variations in riboflavin intake can be at least partially eliminated by collecting evening samples. The urine sample upon rising in the morning should be the most satisfactory since it will ordinarily represent a fairly long collection and be less variable than those collected during the daytime. Johnson et al. ('45) have previously concluded that the values from a urine sample before breakfast were more reliable than those taken during the day.

From the data presented by Brewer et al. ('46) and Horwitt et al. ('50) it would appear that the urinary excretion of riboflavin changes rather markedly when the intake rises above about 1.1 mg per day in the adult subject. Below this level of intake the excretion is approximately 9% of the intake and rises to about 30% of the intake at higher levels of intake. Thus, in a dietary survey one is apparently interested in attempting to determine the number of individuals whose excretion falls below 100  $\mu$ g per day. The creatinine excretion of the average adult (male and female) is approximately 1.2 gm per day. However, for practical purposes we may assume that the critical level is about 100  $\mu$ g per gram

# THE RELATION OF SERUM CHOLESTEROL TO THE PHYSICAL MEASUREMENTS AND DIET OF WOMEN<sup>1</sup>

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A higher incidence of coronary disease and hypertension was observed in the obese (Masters et al., '53). Although it is by no means certain that small differences in the blood cholesterol levels in man influence atherogenesis, hypercholesterolemia appears to be associated with obesity (Gofman and Jones, '52) and cardiovascular atherosclerosis (Gertler et al., '50; Steiner et al., '52; Soffer and Murray, '54). Because most of these observations have been based upon studies of men, an investigation of the serum cholesterol of women in relation to the diet and physical measurements was considered worth while.

The purpose of the present inquiry was to investigate the relationship of varying levels of dietary fat, protein and energy intakes to the serum cholesterol as well as to study its association with the diastolic blood pressure, age and body weight. Additional data of serum cholesterol in healthy women are needed in order to establish values for various age groups.

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in our two-hour samples we would expect at least 11% of the values from a single individual with a mean excretion of 380  $\mu\text{g/gm}$  to be less than 200  $\mu\text{g/gm}$ . Since presumably the data from Newfoundland were skewed similarly to our data,

TABLE 2

*The effect of the number of samples upon the accuracy of the estimated riboflavin excretion*

NO. OF SAMPLES	LENGTH OF COLLECTION PERIOD					
	2 hrs.		4 hrs.		12 hrs.	
	-2 $\sigma$	+2 $\sigma$	-2 $\sigma$	+2 $\sigma$	-2 $\sigma$	+2 $\sigma$
	% of mean		% of mean		% of mean	
1	36	280	41	242	57	175
2	48	208	54	187	67	148
3	55	181	59	169	72	138
4	60	167	65	155	76	132
5	63	158	67	148	78	128
6	66	152	70	142	80	126
10	72 <sup>1</sup>	138	76	132	84	119
15	77	130	80	126	87	116
20	79	126	82	122	88	113
100	90	111	92	109	95	106

<sup>1</sup> Sample calculation:

Mean excretion, 100 $\mu\text{g}$	log 2.0000
Mean + 2 st. dev. = 280 $\mu\text{g}$	log 2.4472
diff., 2 st. dev.	0.4472
1 st. dev.	0.2236
Standard error for mean of 10 samples,	$\frac{0.2236}{\sqrt{10}} = 0.0706$
2.0000 + 2 (0.0706) = 2.1412; antilog = 138%	
2.0000 - 2 (0.0706) = 1.8588; antilog = 72%	

the value of 39% is probably greater than would have been found had logarithms of the data been used. For comparative purposes we have estimated (table 3) the percentage of the samples collected which should be expected to fall below 100 or 200  $\mu\text{g/gm}$  of creatinine at various mean riboflavin outputs when different size samples are used. It should be noted

and free serum cholesterol concentrations when compared with fresh serum samples. The average total and free cholesterol values plus and minus the standard deviations in milligrams percent were respectively: (a)  $193 \pm 34.2$  and  $50.0 \pm 9.41$  for fresh serum, (b)  $190 \pm 37.9$  and  $48.0 \pm 10.5$  after 4 weeks of storage and (c)  $192 \pm 33.5$  and  $45.2 \pm 10.1$ ;  $197 \pm 39.1$  and  $47.2 \pm 9.11$ ; and  $196 \pm 31.8$  and  $45.0 \pm 10.2$  for samples stored 22 weeks and thawed once, twice and thrice, respectively.

*Serum cholesterol for various age groups of women.* Mean values for the total and free serum cholesterol and the ratio of the free to the total cholesterol for all subjects by decades

TABLE 1  
*Total and free serum cholesterol for various age groups of women*

NO. OF SUB- JECTS	AGE			SERUM CHOLESTEROL					
	Range	Mean	S.D. <sup>1</sup>	Total		Free		Ratio of free/total	
				Mean	S.D.	Mean	S.D.		
		yr.		mg %		mg %			
6	< 30	27.3	1.11	207	53.1	51.0	20.3	0.239	0.041
26	30-39	34.5	2.81	211	37.6	53.5	11.1	0.253	0.017
29	40-49	44.6	3.66	250	66.6	64.3	18.9	0.256	0.018
13	50-59	54.2	3.20	293	57.7	78.1	16.4	0.266	0.011
20	60-69	65.8	2.10	302	63.9	79.5	18.5	0.262	0.013
9	≥ 70	77.6	8.85	239	41.1	62.2	10.6	0.261	0.015
113	26-92	48.1	14.9	249	64.3	64.3	18.8	0.256	0.019

<sup>1</sup> The standard deviation of the distribution.

is given in table 1. The ratio of the free to the total serum cholesterol was constant for all age groups. A mean total serum cholesterol value of  $249 \pm 64.3$  mg % was found for 113 women from 26 to 92 years of age. The values range from 141 to 502 mg %.

With increasing age the total serum cholesterol is observed to gradually increase (fig. 1). A significant rise ( $p < 0.01$ ) from  $207 \pm 53.1$  to  $293 \pm 57.7$  mg % at 27.3 and 54.2 years of age respectively is evident. The latter high cholesterol level is maintained until 65.8 years of age. Beyond this age a significant decline ( $p < 0.01$ ) to  $239 \pm 41.1$  mg % at 77.6 years of age occurs.



needed, since average values and the percentage below a given excretion rate do not adequately describe the situation. In future field studies it would be of great advantage to obtain duplicate random samples from each individual. The proportions of the total variance due to differences in individuals (presumably dietary differences or differences in need) and to variation in samples from the same individual could then be determined.

#### SUMMARY

The variation in the excretion of riboflavin per hour, per gram of urinary creatinine, and per milliliter of urine has been studied in several subjects in which urine samples were collected every two or 4 hours.

Regardless of the method of expressing the riboflavin excretion, single urine samples provide a poor estimate of the average excretion rate.

The variation in excretion per hour and per gram of creatinine is of the same order of magnitude while the excretion per milliliter of urine is more variable in most subjects.

The improvement in the estimates of the average excretion obtained by increasing the collection period or by multiple sampling has been calculated. Since the accuracy of the estimate improves as the length of the collection period is increased, field studies should attempt to collect urine over the longest convenient period. The variation from sample to sample is somewhat less in samples collected during the night than during the daytime. Thus, the most valuable sample should be that obtained upon rising in the morning.

The limitations of urinary excretion data in the assessment of the nutritional status with regard to riboflavin for individuals or population groups have been discussed.

#### ACKNOWLEDGMENTS

We wish to acknowledge the cooperation of the staff of the Wrentham State School which made the collections of urine

values has been recorded in the literature for American women. Swanson et al. ('55) studying a similar age group of women found a significantly lower average serum cholesterol concentration. They reported a mean value of 209 mg % of blood cholesterol for 184 women with an average age of 48 years. For women 50 or more years of age our value of 285 mg % of total cholesterol is comparable to that of 270 mg % reported by Gillum and co-workers ('55) for 246 women. A lower mean total serum cholesterol value of 237 mg % for women over 40 years of age (Kountz et al., '45) as well as a higher mean value of 310 mg % for women beyond 61 years of age (Hobson et al., '53) has been reported than is found in comparable age groups of women in the present study who had a mean of 322 and 283 mg % respectively.

*Correlations of the physical measurements with the serum cholesterol.* Since hypercholesterolemia was found in the obese men (Gofman and Jones, '52) and the hypertensive (Stewart and Basu, '51), the tenability of these findings as well as the relationship of serum cholesterol to age was investigated in women.

TABLE 2

*Serum cholesterol levels associated with age, relative body weight and diastolic blood pressure*

NO. OF SUB- JECTS	TOTAL SERUM CHOLESTEROL			AGE		RELATIVE BODY WEIGHT		DIASTOLIC BLOOD PRESSURE	
	Range	Mean	S.D. <sup>1</sup>	Mean	S.D.	Mean	S.D.	Mean	S.D.
	mg %	mg %		YRS.		Lbs./in.		mm of Hg	
17	< 160-189	166	14.7	36.8	11.1	2.02	0.408	78	20.5
20	190-219	203	7.79	41.1	11.0	2.08	0.246	71	9.78
26	220-249	234	8.86	47.0	15.0	2.20	0.363	80	16.2
21	250-279	262	7.10	51.2	15.0	2.25	0.433	82	14.5
12	280-309	293	5.71	56.9	14.4	2.56	0.364	90	13.4
9	310-339	323	10.3	60.3	8.27	2.36	0.558	80	10.3
3	340-369	354	11.0	58.0	8.60	2.45	0.518	108	8.64
5	≥ 370	431	60.4	57.4	8.52	2.18	0.373	92	14.6
113	142-592	249	64.3	48.1	14.9	2.22	0.403	81	16.5

<sup>1</sup> The standard deviation of the distribution.



counted for almost all of the variation in serum cholesterol.

Similar to our findings a positive correlation between the relative body weight and skinfold thickness with hypercholesterolemia, independent of age, was found in a population group of Spanish women by Keys and co-workers ('54). Hobson et al. ('53) also reported this relationship in American women using the skinfold thickness. Neither of these groups of workers, however, was able to demonstrate this positive correlation in men. Yet, Walker ('53) observed in both sexes that overweight was positively associated with elevated serum cholesterol levels. Negligible correlations between body weight and serum cholesterol values for supposedly normal women were reported by Gillum et al. ('55) and Swanson et al. ('55).

Surveys of population groups heretofore have not demonstrated a relationship between the systolic or diastolic blood pressure (Hobson et al., '53; Gillum et al., '55; Swanson et al., '55) with the serum cholesterol.

*Correlations of the diet with the serum cholesterol.* Controlled human experiments have established that a marked increase or decrease in the level of dietary fat causes a corresponding rise or fall of the serum cholesterol (Hildreth et al., '51; Mayer et al., '54; Anderson and Keys, '53). Negative caloric balances without fat restriction inducing weight loss (Pomeranze et al., '54; Walker et al., '53) revealed a significant decrease of serum cholesterol only in those overweight persons with previously high cholesterol values. However, rapid weight gain induced by a high caloric, fat-free diet resulted in a marked rise of initially low serum cholesterol values (Walker et al., '53). Because changes in the serum cholesterol levels appeared to be related to the fat intake as well as to the total calories, the present study investigated by multi-variable analysis the relationship of the total serum cholesterol with the total energy, dietary fat and protein intake. The latter factor was included since protein is known to contain the lipotropic substance methionine.

The percentage of fat and protein calories and total energy for all subjects by 30 mg % intervals of serum cholesterol

ing pigs a 22% corn-solvent soybean oil meal diet containing a 0.31% of methionine and reported that supplemental methionine did not improve the diet. They suggested that the method of processing soybean oil meal might have contributed to apparent discrepancies in reported responses of pigs to methionine supplementation of corn-soybean oil meal diets.

The calculated amino acid composition of 12 to 14% crude protein corn-soybean oil meal diets, based on published values of Baumgarten et al. ('46) and Williams ('55) indicates that such diets may be deficient in tryptophan, methionine and lysine if the presently reported requirements of the pig are accepted. However, Becker et al. ('54) recently reported that diets containing as low as 0.13, 0.23 and 0.63%, respectively, of tryptophan, methionine and lysine were adequate to support satisfactory rate of gain of 40- to 100-pound pigs.

This investigation was conducted to determine the influence upon nitrogen metabolism of growing pigs of supplementing typical corn-soybean oil meal diets containing approximately 12, 14, and 16% of crude protein, and adequate in non-protein dietary factors, with DL-methionine. The nitrogen balance method was employed as it was considered to provide an excellent method by which to measure utilization of dietary protein as affected by methionine supplementation and protein level.

#### EXPERIMENTAL PROCEDURES

Growing barrow pigs of Hampshire or Hampshire  $\times$  Yorkshire breeding and which had been weaned at approximately 8 weeks of age were used in this investigation. The live weight range of the pigs for the duration of the experiment was from 23.4 to 66.5 kg, weights which corresponded to metabolic size values of 10.11 to 21.78. Prior to each preliminary feeding period pigs were randomly assigned to each of the 24 diets used. The test diet was then fed throughout the entire preliminary period and the collection period. All pig weights were reduced to a standard basis of  $W_{kg}^{0.734}$  in an attempt to eliminate insofar as possible the influence of difference in

## SUMMARY

Storage of frozen serum samples for periods of 4 and 22 weeks and thawing of these samples as often as three times did not alter the concentrations of the free and total serum cholesterol when compared with the values in fresh samples.

A mean total serum cholesterol concentration of 249 mg % was observed for 113 women from 26 to 92 years of age. The average cholesterol content of the serum increased gradually from 207 mg % to a maximum of 293 mg % at a mean age of 27.3 and 54.2 years, respectively. This maximum value was maintained during the 6th and 7th decades of life. Thereafter, the concentration of cholesterol declined to 239 mg % at 77.6 years of age. The ratio of the free to total cholesterol was constant for all age groups.

Significant positive correlations for age and relative body weight but a negligible correlation for the diastolic blood pressure with the total serum cholesterol was found. The percentage of dietary fat and protein calories as well as the total energy intake did not show a significant relationship with the variation in total blood cholesterol.

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TABLE 1  
*Composition of basal diets*

INGREDIENTS	AMOUNTS		
	%	%	%
Ground yellow corn	86.25	81.00	75.65
Solvent soybean oil meal	10.70	16.00	21.35
Dicalcium phosphate	1.50	1.50	1.50
Ground limestone	1.00	1.00	1.00
Iodized salt	0.50	0.50	0.50
Vitamin-antibiotic-trace element mixture <sup>1</sup>	+	+	+
Percentage of crude protein	11.8	13.8	15.8
Percentages of amino acids <sup>2</sup>			
Tryptophan	0.10	0.126	0.153
Methionine	0.22	0.25	0.27
Cystine <sup>3</sup>	0.17	0.22	0.26
Lysine <sup>4</sup>	0.58	0.69	0.80
Isoleucine	0.53	0.64	0.74
Histidine	0.30	0.35	0.40
Leucine	1.18	1.32	1.46
Phenylalanine	0.58	0.68	0.79
Threonine	0.45	0.54	0.63
Valine	0.60	0.70	0.81

<sup>1</sup> Provided 2.2 mg of riboflavin, 8.8 mg of calcium pantothenate, 22 mg of niacin, 220 mg of choline chloride, 11  $\mu$ g of vitamin B<sub>12</sub>, 4400 I.U. of vitamin A, 220 I.U. of vitamin D<sub>3</sub>, 22 mg of procaine penicillin, 33 mg of iron, 17.6 mg of manganese, 3.3 mg of zinc, 3.3 mg of copper and 1.1 mg of cobalt per kilogram of diet.

<sup>2</sup> Amino acid assays of basic feed ingredients generously supplied by Dr. Ruth M. Leverton and associates, Human Nutrition Laboratory, University of Nebraska.

<sup>3</sup> Calculated cystine content. (National Research Council, '53.)

<sup>4</sup> The calculated total lysine, including added L-lysine.

nitrogen retention per unit of metabolic size. Data were treated statistically using covariance analysis as set forth by Snedecor ('46).

## RESULTS AND DISCUSSION

Average nitrogen metabolism data for the 11.8, 13.8 and 15.8% crude protein diets are shown in table 2. As is evident from these data, it was only with the addition of 0.1% of DL-methionine to the 11.8% protein diet in the presence of 0.04% of supplemental DL-tryptophan that there was a marked

# THE EFFECT OF RADIATION STERILIZATION ON THE NUTRITIVE VALUE OF FOODS

## I. BIOLOGICAL VALUE OF MILK AND BEEF PROTEINS<sup>1</sup>

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During the past few years considerable interest has been shown regarding the use of ionizing radiations for processing and preservation of foods. Radiant energy at high dosages will not only destroy all microorganisms by inducing changes in their chemical structure but will also bring about chemical changes in the food. These changes may affect the acceptability as well as the nutritive value of the food. Before a new food processing method can be accepted evidence is necessary demonstrating that little or no deleterious change takes place in the nutritive value of the food constituents. Proctor and Bhatia ('50) have reported that the 10 essential amino acids of haddock fillets are not significantly destroyed upon irradiation sterilization. However, the same investigators ('53) have reported that when aqueous solutions of amino acids were exposed to high-voltage cathode rays deamination resulted and the benzene rings in tryptophan, phenylalanine and tyrosine were broken. We have undertaken experiments to investigate the effect of irradiation sterilization of foods on the nutritive value of their proteins and the availability of their energy for the rat. In general the data indicate that

<sup>1</sup> These studies were supported in part under contract no. DA 49-007-MD 544, with the Office of the Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.



influence upon nitrogen retention. Covariance analysis of the data within protein level indicated that there was a highly significant ( $P < 0.01$ ) depression in nitrogen retention of pigs on this particular treatment.

Almquist ('52) reported that the relative proportion of amino acids was a more important attribute than the level of protein in the diet of chicks. If this hypothesis is applicable to swine diets, an imbalance of amino acids may have been created when the 11.8% protein diet was supplemented to provide final levels of at least 0.12 and 0.32% of tryptophan and methionine, respectively. Lysine may have become limiting in this instance as it was present at only 0.58% of the diet. Isoleucine, present at 0.53% of the diet, may have been limiting if the 0.70% of the diet requirement reported by Brinegar et al. ('50b) is accepted as the absolute requirement. However, this explanation is not tenable if the isoleucine requirement is considered to be a function of dietary protein at levels below the optimum. Isoleucine was present at 4.5% of the dietary protein as contrasted to the suggested requirement of 3.2% of the dietary protein by the above workers.

There was considerable variability in the grams of nitrogen retained per unit of metabolic size by the pigs fed the 15.8% protein diet with the various amino acid supplementations, and less variation with the pigs fed the 13.8% protein diet. The differences in grams of nitrogen retained by the pigs on either level of protein and because of amino acid supplementation were not statistically significant. As might have been expected, highly significant ( $P < 0.01$ ) differences resulted in grams of nitrogen retained due to diet, 24 experimental diets being considered. Likewise, highly significant differences in nitrogen retention resulted from the level of dietary protein. The mean nitrogen balance values obtained for pigs fed the 11.8 and 13.8% protein diets were significantly improved through application of within protein level error regression coefficients. The adjusted mean nitrogen balance values are shown in table 2.

losses and for growth) was followed. In all, three experiments were carried out. The first two experiments undertaken on different lots of milk on separate occasions compared the effects of heat and irradiation, respectively, on the nutritive value of the proteins of non-sterilized evaporated milk. The third experiment compared the nutritive value of the proteins of raw and irradiated beef. In each experiment 7 growing rats of the Sprague-Dawley strain were used per treatment. There were three experimental feeding periods in the first test, but only two such periods in the second and the third tests. The experimental diets were fed in periods 1 and 3, while in period 2 all rats received the standardizing diet containing 4% of

TABLE 1  
*Plan of feeding in experiment 1*

PERIOD	GROUP I	GROUP II	GROUP III
1	Non-processed milk	Heat-processed milk	Radiation-sterilized milk
2	4% whole egg diet	4% whole egg diet	4% whole egg diet
3	Radiation-sterilized milk	Non processed milk	Heat-processed milk

whole egg protein. During this second period the ratio of metabolic fecal nitrogen to food consumed, and the ratio of endogenous urinary nitrogen to the three-fourths power of the body weight were determined. The plan of feeding in the first experiment is given in table 1.

Each experimental feeding period lasted 16 days of which the first 9 days were allowed for physiological adjustment to the test diets and during the following 7 days urine and feces were collected. Ferric oxide was used as feces marker.

"Evaporated" milk<sup>1</sup> and heat-sterilized<sup>2</sup> evaporated milk samples were obtained from a commercial source.<sup>3</sup> Beef round steak was purchased from the University Meats Division. After trimming the visible fat, the beef was ground.

<sup>1</sup> See footnote 3, page 480.

<sup>2</sup> See footnote 4, page 480.

<sup>3</sup> Pot Milk Company, Greendale, Illinois.

the response of growing pigs to methionine supplementation in the presence of supplemental tryptophan. This lower protein diet also contained less than 0.7% of isoleucine which could have limited nitrogen retention if the requirement for isoleucine is not a function of protein content of the diet.

Although nitrogen retention values for pigs fed a 15.8% crude protein diet supplemented with several levels of DL-methionine and DL-tryptophan were greater, on the average, than those values obtained for pigs fed a 13.8% crude protein diet supplemented in an identical manner, there was some overlapping of mean nitrogen retention values for the various treatments on the two levels of dietary protein. The addition of 0, 0.025, 0.05 or 0.1% of supplemental DL-methionine to a 13.8% protein diet with or without 0.04% of supplemental DL-tryptophan did not result in significant differences in nitrogen balances of growing swine.

The levels of 0.126, 0.25 to 0.27 and 0.69% tryptophan, methionine and lysine, contained in the 13.8% protein diet, when 0 or 0.025% of supplemental DL-methionine was added, appeared to be adequate to promote satisfactory nitrogen retention by growing pigs fed the diet at approximately 4.00% of their body weights.

The 15.8% protein diet used in these investigations appeared to supply dietary nitrogen in excess of the needs of growing pigs, particularly heavier weight pigs, as evidenced by a marked tendency toward increased loss of nitrogen by urinary excretion.

#### ACKNOWLEDGMENTS

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freeze room (0°F.). The feed jars, one of each diet, were removed from the deep freeze every third day. The jars so withdrawn were stored in a cold room at 40°F., and the diets from these jars were fed to the animals for a period of three days. Left-over diets after three days were discarded, and

TABLE 3  
*Composition of rations*

CONSTITUENT	EVAPORATED MILK DIET	HEAT STERILIZED MILK DIET	IRRADIATION STERILIZED MILK DIET	RAW BEEF DIET	IRRADIATION STERILIZED BEEF DIET
	gm	gm	gm	gm	gm
"Evaporated milk"	36.8 <sup>1</sup>		...	...	...
Heat-sterilized evaporated milk		36.8 <sup>1</sup>	...	...	...
Irradiation sterilized evaporated milk			36.8 <sup>1</sup>	13.6 <sup>1</sup>	...
Raw beef					13.6 <sup>1</sup>
Irradiation sterilized beef	39.7	39.7	39.7	30.0	30.0
Cornstarch				10.0	10.0
Cerelose			10.0	10.0	10.0
Sucrose	10.0	10.0		17.8	18.0
Modified cornstarch <sup>2</sup>				5.1	4.9
Lard				5.0	5.0
Vitaminized cerelose <sup>3</sup>	5.0	5.0	5.0	1.5	1.5
Cod liver oil	1.5	1.5	1.5	0.5	0.5
Wheat germ oil <sup>4</sup>	0.5	0.5	0.5	4.0	4.0
Salts 446 <sup>5</sup>	4.0	4.0	4.0	0.5	0.5
Sodium chloride	0.5	0.5	0.5	2.0	2.0
Wood Flock <sup>6</sup>	2.0	2.0	2.0		
Total	100.0	100.0	100.0	100.0	100.0

<sup>1</sup> On moisture-free basis.

<sup>2</sup> Amidex.

<sup>3</sup> The composition of vitaminized cerelose is as follows: calcium pantothenate 40 mg; pyridoxine hydrochloride, riboflavin and thiamine hydrochloride 5 mg each; nicotinic acid 20 mg; p-aminobenzoic acid 100 mg; inositol 200 mg; choline chloride 2 gm; menadione 2 mg; and cerelose 97.6 gm.

<sup>4</sup> Viobin.

<sup>5</sup> Spector ('48).

<sup>6</sup> Distributed by Brown Company, Portland, Maine.

- SNEDECOR, G. W. 1946 Statistical methods applied to experiments in agriculture and biology, 4th edition. Iowa State College Press, Ames, Iowa.
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of the biological value tests were treated statistically according to the analysis of variance and the results are summarized in table 5.

Apparently the process of evaporation<sup>3</sup> of the raw milk has not altered the biological value of the milk proteins since the biological value of 89.5 for the proteins of the evaporated milk which we obtained compares very well with the value of 89.8 reported by Fairbanks and Mitchell ('35) for raw liquid skim milk. Pasteurization of milk is generally done by holding

TABLE 5  
*Average coefficients of apparent and true digestibility and biological values of protein for the various milk samples*

EXPERIMENT NO.	LIQUID "EVAPORATED" MILK	HEAT STERILIZED EVAPORATED MILK	RADIATION-STERILIZED EVAPORATED MILK	STANDARD ERROR
<i>Apparent digestibility, %</i>				
1 and 2	86.4	85.2	85.3	0.39
<i>True digestibility, %</i>				
1 and 2	97.9	96.7	96.9	0.46
<i>Biological value, %</i>				
1, Period I	92.0	88.2	85.5	0.89
1, Period III	89.1	80.5	77.4	0.59
2	87.2	84.1	82.5	0.97
1 and 2	(89.5)	84.3	81.8	0.53

milk at 143°F. for 30 minutes. Henry and Kon ('36), Krauss ('37), Bixby et al. ('54) and many others have reported data which indicate that pasteurization has little or no effect on the nutritive value of milk proteins. The "evaporated milk," therefore, is as nutritious as raw milk or pasteurized milk as far as the milk proteins are concerned.

*The digestibility of milk proteins.* Detailed statistical analysis of experiment 1 found no appreciable difference between periods or between trios or between rats within trios; these differences were therefore pooled with error. Comparison of error mean squares for the two experiments found them to

<sup>3</sup> See footnote 2, page 480.

taining 14 or 16% of crude protein were not improved for pigs by lysine supplementation. If the values for the lysine content of corn and soybean oil meal reported by Williams ('55) and the National Research Council ('53) are used for purposes of calculation, the diets used by Catron and co-workers would have contained an estimated 0.66 and 0.81% of lysine. Pfander and Tribble ('55) were unable to demonstrate a significant increase in growth rate of growing swine fed 14, 16 and 18% protein corn-soybean oil meal diets due to the addition of 0.1% of L-lysine. They reported that lysine equivalent to 5% of the dietary protein appeared to be adequate for growing swine.

Miner et al. ('55) reported a depression in rate of gain of growing pigs fed a corn-cottonseed meal diet when amounts of DL-lysine in excess of 0.1% of the diet were added. Similar results were observed at this station (Meade, '54) when growing pigs were fed a 14% protein corn-soybean oil meal diet supplemented with 0.15% of L-lysine. The lack of consistency in suggested requirements may indicate that the initially reported requirements were too high. The reported depression in performance due to higher levels of lysine may indicate that balance of amino acids is important. Almquist ('52) has reported that the relative proportion of amino acids is a more important attribute than level of protein in the diet of chicks.

These studies were conducted to determine the influence of lysine supplementation of typical swine diets fed at three levels of protein upon nitrogen utilization by growing swine. The nitrogen balance method has not been widely used in studying amino acid nutrition of growing swine, and it was felt that differences in nitrogen retention of growing swine which might result from lysine supplementation of the diets would help to clarify the lysine requirement. If the addition of an excess of lysine interfered with nitrogen metabolism by disrupting the relative proportion of amino acids in the various diets it should have been reflected in the nitrogen retention values.

tein this means most probably that some essential amino acid has been partially destroyed or bound so as not to be available in the animal and that this amino acid is or has become the limiting amino acid of this treated protein. It may be that due to binding of some sort (e.g. cross link formation) some amino acids are not being released at an optimum rate, causing the amino acid pool to be less efficiently utilized, or are being released in the lower part of the intestine where they undergo alteration due to the flora and, though absorbed, are not available for growth (Carroll et al., '51). Hodson ('54) reported that heat sterilization slightly but significantly lowers the growth-promotive value of evaporated milk and that supplementation with methionine, cystine, and cysteine almost restores the loss of value. Fairbanks and Mitchell ('35) concluded that the proteins of milk are very sensitive to heat treatment. They found that the biological values were lowered by 8%, although the digestibility was not affected when moderate heat processing was used for drying the milk. Supplementation of such dried milk with cystine restored its nutritive value. However, when the temperature of drying in the roller process was increased until perceptible scorching occurred, they observed a further decrease of 12% in the biological value. They state: "the scorched products thus obtained are no longer benefited by cystine additions, but they do respond to lysine additions in increased nutritive value of their proteins." Hence the rapid change in milk proteins at the scorching point (or earlier) is primarily a result of the destruction of lysine. Proctor and Bhatia ('53) measured the ammonia yields from 0.1 M solutions of some amino acids upon irradiation with a cathode-ray dose of 250,000 rep and found that the order of deamination of the amino acids studied was: histidine > cystine > phenylalanine > tyrosine > tryptophan. These investigators ('52) had also observed evolution of hydrogen sulfide upon irradiation of aqueous solutions of cystine thereby indicating that the molecule of cystine was decomposed at the disulfide linkage.



L-Lysine monohydrochloride was added to the diets to provide final lysine levels equivalent to approximately 5 and 6% of the dietary protein. Levels of lysine equivalent to exactly 4% of the diet could not be achieved in the cases of the 14.2 and 16.0% crude protein diets as lysine in excess of 4% of the protein was contributed by the natural feed ingredients. The lower levels of lysine represent 3.96, 4.37 and 4.64% of the protein in the 12.1, 14.2 and 16.0% protein diets, respectively. Each level of lysine feeding was carried out with diets containing zero and 0.04% of additional DL-tryptophan because the 0.11% tryptophan content of the 12.1% crude protein diet was considered to be slightly inadequate, at least for light-weight pigs. DL-Methionine was added to all diets to supply final levels of methionine equivalent to 3.5% of the dietary protein.

The experimental animals used in this investigation were Hampshire  $\times$  Duroc and purebred Duroc barrows which weighed approximately 20 kg at the start of the experiment. Some of the heavier pigs attained weights as great as 58 kg prior to the termination of the experiment. Barrows were randomly assigned to the experimental diets from which the collections were to be made prior to the start of the 8 to 10 day preliminary feeding period and no consideration was given to the pig's previous treatment.

All animals were fed twice daily at a constant level of feed intake closely approximating 4% of body weight throughout the final 4 days of the preliminary feeding period and the entire collection period. Nitrogen balance trials were conducted using 6-day collection periods. Three separate total fecal and urine samples were collected for pigs fed each experimental diet. Cylindrical metabolism cages patterned after those designed by Bell ('48) were used for making collections of excreta.

All data have been summarized to present animal weights as mean weight,  $\bar{W}$ , expressed in kilograms for the time that the individual pigs were on urine and fecal collections. The mean weights of the animals have been raised to the power

These investigators found no significant difference in the digestibility or biological values, the percent true digestibility of the raw beef nitrogen being 99.6 and that of the roast beef, 99.3, and the percent biological values of the raw and roast beef proteins being 73.8 and 74.8, respectively.

#### SUMMARY AND CONCLUSIONS

The effect of irradiation sterilization and of heat sterilization on the nutritive value of milk proteins and beef proteins has been studied by the Thomas-Mitchell method. A 3 million rep gamma irradiation was used to sterilize the frozen milk and beef. The ground beef was vacuum packed during irradiation treatment and milk was conventionally canned. Upon irradiation milk developed a reddish tinge, and the proteins had coagulated; the beef looked somewhat darkened. Irradiation did not produce pronounced off-flavors and odors in beef or milk. The irradiated beef when incorporated into a balanced diet was found completely acceptable to the rat while the diet containing irradiated milk was consumed reluctantly.

Irradiation sterilization did not affect the apparent or true digestibility of the beef (100%) or of the milk protein (98%). The biological value of the beef protein (78%) also was not affected upon irradiation but the biological value of the milk proteins (90%) was reduced by 8% upon irradiation as compared to a reduction of 6% due to heat sterilization. The possibility of irradiation damage to the sulfur amino acids is discussed.

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TABLE 2

*Average nitrogen metabolism data of pigs fed a 12.1, 14.2, and 16.0% crude protein diet supplemented with L-lysine*

PERCENT OF AVAILABLE TRYPTO- PHAN IN DIET	LYSINE CONTENT AS PER CENT OF PROTEIN	MEAN BODY WT. G.	AV. DAILY IN- CREASE	AIR DRY FED CON- SUMED DAILY	NITROGEN METABOLISM PER DAY <sup>1</sup>				NITROGEN METABOLISM PER DAY PER W 0.731			
					Intake	Feces <sup>2</sup>	Urine	Balance	Absorbed	Urine	Balance	
												gm
12.1% crude protein												
0.11	3.96	43.77	0.47	1724	33.42	8.14	11.16	14.12	1.57	0.69	0.85 <sup>2</sup>	
	4.95	33.75	0.38	1370	26.78	8.06	7.65	11.07	1.40	0.57	0.85 <sup>2</sup>	
	5.94	34.16	0.52	1383	27.25	7.14	9.94	10.17	1.50	0.74	0.77 <sup>2</sup>	
0.13	3.96	37.23	0.50	1530	29.74	7.72	10.98	11.04	1.55	0.78	0.77 <sup>2</sup>	
	4.95	29.25	0.44	1185	23.24	6.29	7.23	9.81	1.43	0.61	0.95 <sup>2</sup>	
	5.94	39.65	0.48	1585	31.34	6.97	12.64	11.73	1.58	0.85	0.71 <sup>2</sup>	
14.2% crude protein												
0.137	4.37	40.56	0.53	1611	36.56	8.66	13.63	14.27	1.84	0.88	0.96	
	4.94	36.58	0.57	1419	32.33	8.01	11.92	12.40	1.73	0.82	0.91	
	5.92	40.59	0.59	1616	37.42	8.28	10.58	18.56	1.94	0.71	1.23	
0.157	4.37	38.48	0.49	1537	34.94	8.03	12.21	14.70	1.86	0.85	1.01	
	4.94	35.94	0.45	1432	32.70	6.90	11.77	14.03	1.86	0.85	1.01	
	5.92	36.32	0.47	1444	33.57	7.45	10.07	16.05	1.87	0.72	1.15	
16.0% crude protein												
0.159	4.64	33.14	0.50	1328	33.92	7.52	12.07	14.33	2.01	0.92	1.09	
	5.01	32.98	0.53	1321	33.84	8.04	10.48	15.32	1.99	0.80	1.19	
	6.02	43.92	0.64	1785	46.09	10.25	18.10	17.74	2.22	1.08	1.14	
0.179	4.64	40.14	0.67	1641	42.01	10.38	14.54	17.09	2.09	0.95	1.14	
	5.01	44.87	0.69	1737	44.58	11.98	14.78	17.82	2.00	0.90	1.10	
	6.02	43.92	0.44	1713	44.32	10.62	15.00	18.70	2.10	0.94	1.16	

<sup>1</sup> Mean values for three pigs per treatment.  
<sup>2</sup> Apparently absorbed nitrogen.

<sup>1</sup> Mean values for three pigs per treatment.

<sup>2</sup> Apparently absorbed nitrogen.

<sup>3</sup> Adjusted mean values obtained by application of within protein level error regression coefficient.

# THE INFLUENCE OF CREATINE BIOSYNTHESIS ON THE ARGININE REQUIREMENT OF THE CHICK<sup>1</sup>

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Arginine has been established as an essential amino acid for the chick (Klose et al., '38). Working with 4-week-old chicks, Almquist and Merritt ('50) found the arginine requirement for optimum growth to be 1.2% of a semi-purified diet containing 20% protein. On the other hand, several recent investigations (Young et al., '53; Wietlake et al., '54; Snyder et al., '54) employing purified diets have demonstrated that this level of arginine is insufficient for the day-old chick. In an attempt to explain this discrepancy on the basis of dietary differences among workers, Griminger et al. ('55) examined the possibility of an arginine loss or non-availability in completely purified diets as a factor causing an apparent increase in the arginine requirement. This postulation, however, was not substantiated, for the arginine requirement was again shown to be significantly greater than 1.2% under conditions which prevented any possible loss of arginine from the diet.

It has been shown in this laboratory that muscle creatine, for which arginine serves as a precursor, increases linearly for the first 4 weeks of the chick's life (Fisher et al., '56). Wietlake et al. ('54) have also found that in day-old-chicks dietary creatine will exert a sparing effect on arginine. In

<sup>1</sup> Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Departments of Poultry Husbandry and Agricultural Biochemistry, New Brunswick

as 0.137, 0.62 and 0.63% of tryptophan, lysine and isoleucine, respectively. The 0.48% of methionine contained in the 14.2% protein diet is considered to be in excess of the pigs' needs since a calculated excess of methionine was intentionally added to the diet.

Nitrogen retention values of pigs obtained when the two higher protein diets were fed do not indicate that increasing levels of lysine had a depressing effect upon nitrogen retention. The relative proportion of lysine to other essential amino acids did not remain constant at the two higher levels of protein. These results indicate that amino acid requirements may be a function of protein content of the diet and that relative proportion of amino acids may be important until an adequate amount of dietary protein is included in the diet.

#### SUMMARY AND CONCLUSIONS

A 12.1% crude protein diet was inadequate to support satisfactory nitrogen retention by growing pigs, due in part to inadequate total nitrogen intake. This diet should have been adequate in tryptophan, methionine and lysine, after supplementation. This diet contained less isoleucine than has been reported to be required by growing pigs.

Nitrogen retention values ranging from 0.96 to 1.23 gm of nitrogen retained per unit of metabolic size resulted when a 14.2% protein corn-soybean oil meal diet containing 0.137 to 0.157, 0.62 to 0.84 and 0.63% of tryptophan, lysine and isoleucine, respectively, was fed to growing pigs.

The use of higher levels of lysine in conjunction with a 12.1% crude protein diet appeared to contribute toward a decrease in nitrogen retention of growing pigs, perhaps because lysine was not in the correct proportion to other essential amino acids in the diet. Supplementation of higher protein diets with additional lysine when these diets apparently contained adequate amounts of all other essential amino acids did not significantly influence nitrogen balance of growing pigs.

# ARGININE REQUIREMENT OF THE CHICK

TABLE 1  
Composition of rations

BASAL DIET			CHICK STARTER	
Ingredients	Amount	Vitamins added	Ingredients	Amount
				Per 100 lbs.
	%			
Corelose	61.06	Thiamine HCl	Conc meal—lb.	53.6
Caseln (crude)	25.00 <sup>1</sup>	Riboflavin	Soybean meal (50%)—lb.	34.0
Salt mixture <sup>2</sup>	5.34	Calcium pantothenate	Alfalfa—lb.	3.0
Corn oil	3.00	Niacin	Corn distillers solubles—lb.	4.0
Non nutritive fiber	3.00	Pyridoxine HCl	Corn distillers—lb.	1.0
Choline chloride	0.20	Biotin	Butyl fermentation—lb.	0.5
A & D (10,000 A-6000 D <sub>2</sub> )	0.10	Folic acid	B <sub>12</sub> -antibiotic supplement—lb.	2.2
Glycine	2.00	Inositol	Dicalcium phosphate—lb.	1.0
DL-Methionine	0.30	Para-aminio benzoic acid	Mineral concentrate—lb.	0.5
Vitamins	+	Menadione	Salt—lb.	96 gm
	100.00	$\alpha$ -Tocopherol acetate	Choline chloride (25%)	1 gm
		Ascorbic acid	Niacin	0.2
		Vitamin B <sub>12</sub>	A & D (1500 A-300 D <sub>2</sub> )	
				0.02

<sup>1</sup> N = 0.25 = 20.1%.

<sup>2</sup> For composition see Fisher et al. (1954).

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necessary to ascertain what level of dietary creatine would yield optimum growth and muscle creatine in the absence of supplemental arginine (the basal diet contained approximately 0.8% of arginine). As shown in table 3, the levels of creatine ranging from 0 to 4% were fed, and weight gains, feed utilization and muscle creatine levels recorded at the end of three weeks. It can be seen that 1% of creatine was optimum in supporting growth and feed utilization on this diet. This, however, was not as good as could be obtained in the presence of adequate arginine. That 0.8% of arginine (present

TABLE 3

*Effect of dietary creatine on growth and muscle creatine in the chick*

DIETARY LEVEL OF CREATINE HYDRATE	AVERAGE OF DUPLICATE GROUPS AT 3 WEEKS <sup>1</sup>				
	Gain		Gain/feed	Muscle creatine	
%	gm	S.D. <sup>2</sup>	C.V. <sup>4</sup>	mg/gm	S.D. <sup>2</sup>
0 (basal) <sup>2</sup>	118 ± 21	(56)	0.448	2.63 ± 0.05	
0.5	172 ± 17	(32)	0.532	3.42 ± 0.05	
1.0	196 ± 13	(21)	0.609	3.59 ± 0.14	
2.0	208 ± 12	(18)	0.586	3.75 ± 0.11	
4.0	183 ± 14	(24)	0.517	4.11 ± 0.09	

<sup>1</sup> Five birds per group.

<sup>2</sup> Containing 0.8% arginine.

<sup>3</sup> ± standard deviation of the mean.

<sup>4</sup> Coefficient of variation.

in the basal) was still limiting, even with 4% of supplemental creatine, was demonstrated by the upturned and frizzled feathers of all groups of birds which in our experience is characteristic of an arginine deficiency. The arginine deficiency is further reflected in the high coefficients of variation (table 3), an observation previously made in this and other laboratories (Wietlake et al., '54; Fisher et al., '56). Muscle creatine increased with every increment in dietary creatine. In view of the latter finding the results of experiment 2 can be interpreted to mean that some arginine is being diverted for creatine biosynthesis even when the muscle creatine content is already high or "normal." This would then explain



basal "milk-type" diet similar to that used in the studies of the leucine requirement of the suckling pig (Eggert et al., '54), which is capable of supporting normal growth of young pigs, would be low enough in histidine content to be useful for a quantitative study of the histidine requirement. Supplementary amounts of the 10 amino acids known to be required for the growth of the weanling rat were added to one diet, while the same amino acids minus histidine were supplied to the other diet. One per cent of monosodium glutamate was supplied in each diet and sufficient diammonium citrate was added to equalize the nitrogen content of each diet at a level equivalent to 20% of the air-dry diet as crude protein ( $N \times 6.25$ ) instead of the 25% level used in the leucine experiments.

Eight litter-mate Yorkshire pigs were removed from the sow at two days of age. They were fed a stock diet containing 25% of protein (casein) for 5 days, at which time they were divided into two groups on the basis of weight and sex, and shifted gradually over the course of 4 feedings to the two experimental diets. All pigs were individually fed essentially ad libitum, and weight gains measured at weekly intervals for a 21-day period were used as the criteria of judging the adequacy of the experimental diet.

*Experiment 2.* In order to reduce the attending difficulties encountered with a liquid diet, dry diets were used in experiments 2 and 3.

Eight Berkshire  $\times$  Yorkshire pigs were removed from the sow at 10 days of age and placed in a heated battery unit. For the initial 7-day period they were fed a low-fat, dry feed mixture described by Crampton and Ness ('54) with some modifications. Then they were divided on the basis of weight into two replicates of 4 each, and randomized to treatments and pens. The treatments were 4 levels of histidine; 0.1, 0.2, 0.3, and 0.4% of the total diet. The basal experimental ration was compounded to provide 20% protein equivalent, 10% fat and 2% fiber. It consisted of 33.33% dried whey, 4.45% amino acid mixture, 12.32% diammonium citrate, 13.00% glucose, 25.23% dextrinized starch, 9.67% corn oil and 2.00% fiber source.

## DISCUSSION

An unusual feature of the data on arginine requirement is the growth plateau which occurs before the arginine level for maximum growth and feed efficiency has been reached. This plateau, which is *not* considered an artifact in view of its recurrence in each replicate and experiment, makes the statistical evaluation of a maximum response a difficult one. Regression analysis is complicated by the plateau and "t" test or least significant difference analyses are not necessarily apropos with dose-response data as pointed out recently by Almquist ('54).<sup>3</sup> Confirmation of the validity for this unusual growth plateau can be found in the arginine studies of Snyder ('54). He obtained the same plateau starting at the 1.3% arginine level, followed by a further increase in growth at higher arginine levels. He states the requirement to be 1.7%. This unique curve for arginine may be analogous to the lysine response curve of *S. fecalis* first obtained by Stokes et al. ('45).

The present data would indicate that the arginine requirement for the first three weeks of life on casein diets is greater than 1.3% and that for *maximum* growth and efficiency, dietary creatine will spare but a small amount of arginine.

The finding that some arginine is always being diverted for creatine synthesis parallels the observation by Fisher et

<sup>3</sup>"By the usual statistical tests it may be found that at the lower levels of F the response,  $\Delta R$ , to a given  $\Delta F$  is "significant." This significance is usually expressed as some ratio between the  $\Delta R$  and the probable error, or standard error of  $\Delta R$ , etc. As F increases, however,  $\Delta R$  may become steadily smaller in reference to a given  $\Delta F$ , according to the diminishing returns principle. It should be pointed out that this tendency alone may be sufficient to cause an application of the statistical tests for significance of difference to indicate that at higher levels of F observed,  $\Delta R$  is no longer "significant." As a result, the investigators may conclude that there is no difference in R between two levels of F being tested near the top of the response curve. Thereupon, it may be decided that the lower level of F, of the two being compared, is as satisfactory as the higher level in meeting the full requirements of the animal. Because of this systematic error in interpretation, arising from disregard of the diminishing returns principle where it applies, the nutritional requirements of animals have been subjected to a tendency toward underestimation."

dom into 4 groups of 4, in such a way that each group contained one pig from each litter, and placed on the experimental diets.

TABLE 2

*Amino acid composition and nitrogen content of dried whey and amino acid mixture, used in experiment 3*

	AMOUNT SUPPLIED BY				TOTAL AVAILABLE AMINO ACID OF BASAL DIET	
	Whey <sup>1</sup>	Amino acid mixture		N content		
		Form				
	%	%		%	% of diet	% of crude protein <sup>2</sup>
Arginine	0.06	0.22	L-HCl	0.06	0.24	1.5
Histidine <sup>3</sup>	0.03		L-HCl		0.03	0.2
Isoleucine	0.16	0.80	DL	0.09	0.56	3.5
Leucine	0.26	0.54	L	0.06	0.80	5.0
Lysine	0.22	0.73	L-HCl	0.11	0.80	5.0
Methionine <sup>3</sup>	0.03	0.39	DL	0.04	0.42	2.6
Phenylalanine <sup>3</sup>	0.07	0.43	DL	0.04	0.50	3.1
Threonine	0.14	0.84	DL	0.10	0.56	3.5
Tryptophan <sup>3</sup>	0.03	0.13	DL	0.02	0.16	1.0
Valine	0.13	0.70	DL	0.08	0.48	3.0
Cystine <sup>4</sup>	0.06				0.06	0.4
Tyrosine <sup>4</sup>	0.06				0.06	0.4
Total	1.25	4.78		0.60	4.81	29.2

<sup>1</sup> Per cent of amino acid  $\times$  % digestibility of whey protein (90%). Figures for the amino acid composition of dried whey were obtained from Williams ('55) and figure for the digestibility of whey protein was obtained from Morrison ('51).

<sup>2</sup> The histidine content of dried whey was determined by column chromatography (Moore and Stein, '51).

<sup>3</sup> Methionine, phenylalanine and tryptophan are utilized in both forms.

<sup>4</sup> Cystine has a sparing action on methionine and tyrosine on phenylalanine.

<sup>5</sup> Crude protein = 16.0% of diet.

The basal diet, shown in table 1, contained 16.0% total crude protein, 2.77% being supplied by dried whey ( $N \times 6.44$ ),<sup>1</sup> 3.75% by amino acids ( $N \times 6.25$ ) and 9.48% by diammonium citrate ( $N \times 6.25$ ). The nitrogen and amino acid contents of the amino acid mixture are summarized in table 2. The dietary contents of the individual amino acids were based on values which had been determined for baby pigs, where available.

<sup>1</sup> The N factor of 6.44 was suggested by Perlmann and Longworth ('48) and is based on  $\beta$ -lactoglobulin, the main constituent of whey protein.

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not much, if any, higher than 0.3% of such a diet. This would be equivalent to a requirement of approximately 1.5% of the dietary protein.

The results of the second experiment are summarized in table 4. The 0.3% level of total L-histidine appeared to be optimum and 0.2% produced gains of essentially the same magnitude. The 0.1% level was associated with a less uniform response of a lesser magnitude. In terms of both gains and appetite of the pigs the 0.4% level was poorest. When the

TABLE 4  
*Average data for pigs receiving various levels of histidine*  
Experiment 2

	SUPPLEMENTAL LEVEL OF DL-HISTIDINE (%)			
	0.06	0.26	0.46	0.66
Total L-histidine, % of diet <sup>1</sup>	0.10	0.20	0.30	0.40
Total L-histidine, % of protein	0.50	1.0	1.50	2.00
Number of pigs	2	2	2	2
Days on trial	18	18	18	18
Initial weight, kg	3.8	3.6	3.5	3.6
Final weight, kg	4.5	4.4	4.4	3.8
Daily gain, gm	35 ± 7 <sup>2</sup>	46 ± 2	51 ± 7	11 ± 24
Daily gain, % of initial weight	0.91	1.25	1.48	0.32

<sup>1</sup> See footnote 2, table 3.

<sup>2</sup> Standard deviation.

pigs in lot 4 were transferred to the 0.3% level for an additional 5 days, they gained rapidly, the average daily gain being 115 gm. The abrupt gain in one pig from lot 4 in the three days prior to changing the ration cannot be explained.

A summary of the data obtained in the third experiment is presented in table 5. That the basal diet is deficient in histidine (0.03% of the diet) is indicated by the slow growth, poor appetite and low feed utilization. Increasing the level of histidine in the diet improved the pigs' appetites and produced significantly higher daily gains. In terms of growth, appetite and efficiency of feed utilization the 0.19% level of total L-histidine appears to be approximately optimum. However, the

# A COMPARISON OF THE BIOLOGICAL VALUES OF DIETARY PROTEIN INCORPORATED IN HIGH- AND LOW-FAT DIETS

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The thesis has been generally accepted that the chief function of carbohydrate or fat in the animal organism is calorigenic and that dietary carbohydrate is better than dietary fat as a protein-sparing nutrient. Even excessive amounts of fatty acids are oxidized through the tricarboxylic cycle in the presence of oxalacetic acid and in this way fatty acids of the dietary fat would become available for energy purposes. However, if a diet consists solely of fat, large amounts of ketone bodies would be produced and the kidney would probably produce more ammonia in order to neutralize these acids. The result may be an increased catabolism of the tissue protein. Literature on protein utilization provides conflicting evidence regarding the effect of dietary fat on protein metabolism. The discrepancies among the results of various laboratories seem, in part, due to ineffective experimental techniques or to dubious interpretation of the data. Many research workers have used growth of the young animal or repletion of the protein-depleted animal to measure the nutritive value of a protein. The nitrogen balance index method of Allison and Anderson ('45) or the biological value method of Mitchell ('24) are among the more precise methods for measuring even small differences in protein quality.

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A comparison of the last two experiments suggests that the D form of histidine is poorly utilized, if at all, when it is fed in a racemic mixture, since in experiment 2 the DL form was fed but the response was proportional to the level of the L form as judged by the results of experiment 3. Similar findings were reported in studies with dogs (Abderhalden and Buadze, '31), guinea pigs (Edlbacher et al., '41), rabbits (Abderhalden, '22), man (Albanese et al., '45) and the mouse

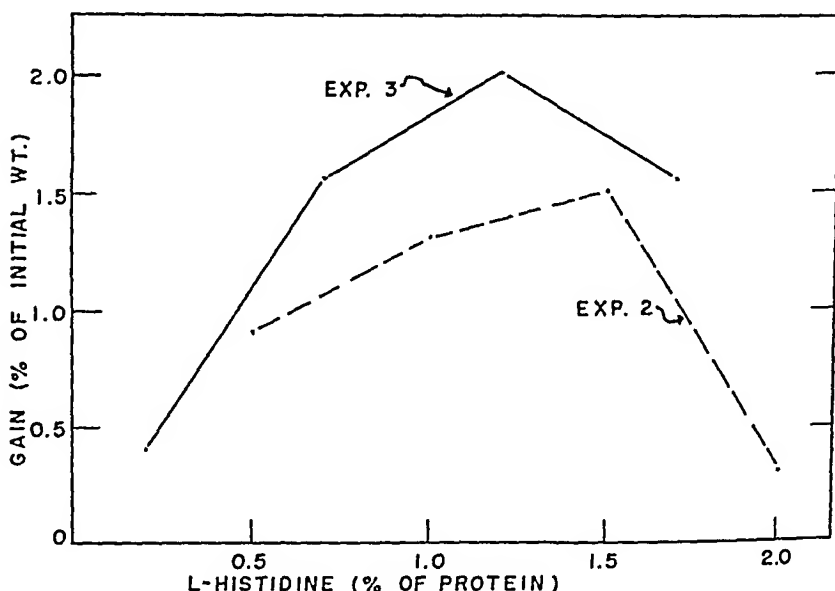


Fig. 1 Relative growth response of baby pigs fed different levels of L-histidine.

(Celandier and Berg, '53). However, the rat seems to utilize the unnatural isomer both for growth (Cox and Berg, '34) and for the maintenance of nitrogen equilibrium (Nasset and Gatewood, '54).

It is seen that the daily gains in the first experiment were much higher than those in the latter two experiments. It seems probable that the liquid nature of the first diet was not entirely responsible for the greater gains, the dietary components of that diet could have been a factor. It contained

nitrogen) diets (III and IV, also low or high in fat). During the third experimental period reversal feeding of the test diets was followed so that the group which had received the low-fat diet in the first period now received the high-fat diet, and vice versa. The adequacy of a 10-day pre-feeding period

TABLE 1  
*Composition of the experimental diets*

CONSTITUENTS	TEST DIETS		STANDARDIZING DIETS		TEST DIETS	
	Diet I	Diet II	Diet III	Diet IV	Diet V	Diet VI
	%	%	%	%	%	%
Whole egg <sup>1</sup>			5.00	6.67		
Casein <sup>2</sup>	12.00	16.00			10.00	13.33
Salts 446 <sup>3</sup>	4.00	5.33	4.00	5.33	4.00	5.33
Sodium chloride	1.00	1.33	1.00	1.33	1.00	1.33
Vitaminized starch (691) <sup>4</sup>	5.00	6.67	5.00	6.67	5.00	6.67
Liver fraction (Wilson 1:20)	0.25	0.33	0.25	0.33	0.25	0.33
Cod-liver oil	1.50	2.00	1.50	2.00	1.50	2.00
Wheat germ oil	0.50	0.67	0.50	0.67	0.50	0.67
Wood flock <sup>5</sup>	2.00	2.67	2.00	2.67	2.00	2.67
Carbohydrate mixture <sup>6</sup>	70.75	34.33	77.82	44.39	72.75	37.00
Lard	3.00	30.67	2.93	29.94	3.00	30.67
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00

*Analysis of diets after mixing (grams per 100 gm)*

Total nitrogen	1.75	2.33	0.61	0.82	1.47	1.93
Ether extract	4.89	32.71	5.20	33.57	5.12	33.41
Dry matter	92.87	95.81	93.59	94.81	92.68	95.76
Gross energy (calories)	401	558	391	545	398	553
Isocaloric factor	1.00	0.75	1.00	0.75	1.00	0.75

<sup>1</sup> Dried and ether-extracted.

<sup>2</sup> Labco, vitamin-free.

<sup>3</sup> Spector ('48).

<sup>4</sup> The percentage composition of vitaminized starch is as follows: calcium pantothenate 40 mg; pyridoxine hydrochloride, riboflavin and thiamine hydrochloride 5 mg each; nicotinic acid 20 mg; *p*-aminobenzoic acid 100 mg; inositol 20 mg; choline chloride (Merck dry mix) 8 gm; and cornstarch 91.525 gm.

<sup>5</sup> Distributed by Brown Co., Portland, Maine.

<sup>6</sup> Carbohydrate mixture consisted of cornstarch, cellulose and sucrose in the proportions of 5:3:1.



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TABLE 2  
Effect of fat level on the digestibility and utilization of dietary nitrogen  
(average daily data of 7 rats with standard errors)

DIET	NITROGEN INTAKE	DRY MATTER INTAKE	APPARENT DIGESTIBILITY OF CASEIN	TRUE DIGESTIBILITY OF CASEIN	NITROGEN BALANCE	BIOLOGICAL VALUE
	mg	gm	%	%	mg	%
<i>Experiment on severely protein-depleted rats</i>						
<i>Period I</i>						
I (low fat)	210	11.51	89.2 ± 0.02	98.2 ± 0.51	89.3 ± 1.63	67.2 ± 0.33
II (high fat)	210	8.33	89.2 ± 1.56	97.2 ± 0.85	88.2 ± 2.81	68.0 ± 0.43
Mean difference		3.18		1.0	1.1	0.8
P				0.3 < P < 0.4	0.7 < P < 0.8	0.6 < P < 0.7
<i>Period III</i>						
II (high fat)	209	8.33	90.1 ± 0.07	98.7 ± 0.99	76.8 ± 1.3	64.4 ± 0.91
I (low fat)	209	11.51	89.4 ± 0.46	98.4 ± 1.07	85.9 ± 3.1	69.5 ± 1.75
Mean difference			1.0	0.3	9.1	5.1
P			0.05 < P < 0.1	P = 0.8	0.01 < P < 0.02	0.02 < P < 0.05
<i>Experiment on growing rats</i>						
VI (low fat)	125	7.88	90.2 ± 0.27	99.5 ± 0.27	57.5 ± 1.1	72.4 ± 1.2
VII (high fat)	123	6.11	90.9 ± 0.28	99.6 ± 0.18	55.0 ± 0.9	69.2 ± 0.8
Mean difference		1.77	0.7	0.1	2.5	3.2
P			0.05 < P < 0.1	0.6 < P < 0.7	0.1 < P < 0.2	0.05 < P < 0.1

this reason, the combined effect of other substances with vitamin D could not be evaluated.

3. Proportionally more of the absorbed  $\text{Ca}^{45}$  was found in the femurs of the vitamin D-supplemented rat than in the vitamin D-deficient rat. Vitamin D had no apparent effect on the deposition of absorbed  $\text{Ca}^{45}$  in the tibias of the rachitic chick.

#### ACKNOWLEDGMENTS

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# MILLET (*SETARIA ITALICA*): ITS AMINO ACID AND NIACIN CONTENT AND SUPPLE- MENTARY NUTRITIVE VALUE FOR CORN (MAIZE) <sup>1</sup>

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## INTRODUCTION

"Millet" is a generic term applied to any of various small seeded cereals and forage grasses and frequently used in some countries as a general designation for sorghum, wheat or other native cereals. Botanically, distinct species are regarded as millets, the most common of which are *Panicum miliaceum* (common millet), *Setaria italica* (Italian millet), *Eleusine coracana* (finger millet or ragi), *Pennisetum typhoides* (pearl millet) and *Echinochloa crusgalli* (Japanese barnyard millet). Anderson and Martin ('49) detail the macroscopic structure and characteristics of these various species of millet.

Millet is often considered to be a "poor man's cereal." In some regions of Eastern Europe and Africa it is consumed in the form of porridge or flatbread. In the United States it is used mainly in poultry feed. Few studies have been made of the nutritive value of this cereal. Data concerning the

\* A portion of these data are taken from a thesis submitted to Vanderbilt University by one of the authors (A.S.M.) in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry.

<sup>1</sup> This study was supported in part by grants from the Williams-Waterman Fund for the combat of dietary diseases and from the H. I. du Pont de Nemours and Company.

ably fortified halibut liver oil was added to the ration once each week to provide the following intakes: vitamin A, 400 I.U.; vitamin D, 4 I.U.; 2-methyl-1, 4-naphthoquinone, 0.06 mg; and alpha-tocopherol, 0.7 mg. The supplements studied (maize, millet, niacin, tryptophan and lysine) were incorporated at the expense of sucrose and at the levels indicated in tables 2, 3, 4, 5 and 6.

The total nitrogen content of millet and maize was determined by the macro-Kjeldahl method (Association of Official Agricultural Chemists, '50). The estimate of protein content was made by multiplying the per cent nitrogen in a sample by 6.25. Moisture was determined by drying the sample to constant weight at 110°C.

The amino acid composition of millet and maize was determined microbiologically. Tryptophan was estimated by the procedure of Greene and Black ('44), and niacin was determined by a modification of the method of Snell and Wright ('41). Both of these assays were made at the conventional 10 ml assay level. Assays for other amino acids (lysine, tyrosine, phenylalanine, methionine, valine, leucine, isoleucine, arginine and cystine) were carried out in a total volume of 2 ml according to the procedure of Snell ('45). Commercial assay media<sup>7</sup> were used in the determination of all amino acids except tryptophan, threonine, and histidine. The latter amino acids were determined using *Streptococcus faecalis* 29-21<sup>8</sup> and *Lactobacillus arabinosus* ATCC 8014, respectively, according to the procedure of Stokes et al. ('45).

#### RESULTS AND DISCUSSION

The amino acid and niacin contents of millet and maize used in the present investigations are given in table 1, along with values from the literature for whole wheat, ragi and Italian millet. The niacin content of millet found in this study (1.40 mg %) is similar to that reported by Aykroyd and

<sup>7</sup> Difco Laboratories, Detroit, Michigan and H and M Chemical Company, Santa Monica, California.

<sup>8</sup> Hartman, A. P., and P. A. Hanson, J. Bact., 59: 197, 1957.

Swaminathan ('40). However, later studies by Swaminathan ('44), indicated a much lower (0.7 mg %) niacin content of the same kind of millet and Giri and Nagana ('41) reported that Italian millet does not contain measurable amounts of this vitamin. Our analyses indicate a niacin content of millet somewhat lower than that of maize and wheat and approximately that found in ragi (finger millet). The variability in results obtained by these investigators may be due to different methods of determination. Giri and Nagana ('41) and Swaminathan ('44) used a chemical method of analysis, while Aykroyd and Swaminathan ('40) employed a microbiological method of determination.

The amino acid values obtained in this study and the results of Baptist and Perera ('56) are compared in table 1. With the exception of leucine and isoleucine, the values reported by these workers are somewhat higher than ours. Whether this discrepancy reflects actual differences in the amino acid content of the samples assayed or differences in the techniques employed cannot be stated. However, both analyses reveal a similar amino acid distribution pattern with lysine being the most limiting amino acid. The nutritional significance of the relatively high tryptophan content of Italian millet will be discussed later. In general, the assay values presented here tend to confirm the relatively high biological value of this cereal as reported by others (Niyogi et al., '34; Swaminathan, '37; Mukjerjee and Parthasarathy, '48).

A few studies of the amino acid composition of ragi (finger millet) are available and the data from the publications of Lal ('50), Balasubramanian, et al. ('52) and of Baptist and Perera ('56) are included in table 1. On the basis of the analytical data, Italian millet is clearly superior to ragi as a protein source. Except for lysine, the amino acid composition of the former is superior in every case. The amounts of tryptophan, leucine, histidine, isoleucine and phenylalanine found in Italian millet are approximately three times those

permit selection of the variety of this cereal best suited for human and animal feeding.

In comparing the amino acid composition of Italian millet with that of maize, it is seen that this millet contains twice as much tryptophan as does maize, but is more limited in lysine, tyrosine, arginine and histidine. In figure 1, the proportions of amino acids of millet and maize are compared with those reported by Flodin ('53) as optimum for growth of the rat. On this basis, Italian millet appears to be most deficient in lysine, but is probably also inadequate in histidine, threonine, valine and methionine and cystine. Despite the limitations of this method of assessment, the nutritional weakness of this cereal as a sole source of protein is evident. The inadequacy as predicted from these analyses has been confirmed, and will be discussed later.

The total protein content of Italian millet is higher than that of maize or ragi, but slightly lower than wheat. The discrepancy in the protein content of ragi as reported by Lal ('50) and by Baptist ('54) requires clarification.

Data on the growth-promoting value of maize and millet are shown in table 2. These data are for rats on a basal diet containing 9% casein. All results are expressed as total weight gains and protein efficiency ratios.<sup>9</sup> The control animals fed the basal diet + 40% maize grew poorly, with a resulting total weight gain of but 40 gm and a protein efficiency ratio of 1.40 during the experimental period of 29 days. In this experiment, the growth was less than that obtained on the basal diet alone. Supplementing the ration with 3 mg% of niacin enhanced both the growth and protein efficiency ratios to 108 gm and 2.42, respectively. To compare the supplementary value of millet to that of maize, rats were fed the basal (9% casein) diet + 40% millet. In contrast to the maize diet, this ration satisfactorily supported growth with a total weight gain of 109 gm and a protein efficiency

<sup>9</sup> Protein Efficiency Ratio (P.E.R.)

$\frac{\text{grams gain in body weight}}{\text{grams protein consumed}}$

efficiency ratio was less than that found with the 40% millet diet or 40% maize + niacin diet.

The growth produced by basal diet + 40% maize + 40% millet was equivalent to that produced by the basal-maize diet + niacin. The addition of niacin to basal + maize + millet diets improved neither growth nor the protein efficiency ratio. Reducing the amount of maize to 20% in the diet did not significantly affect the growth of rats. However, the protein efficiency ratios produced by diets containing 20% maize were slightly higher than those containing 40% of this cereal.

Since supplementation of the maize diets with either niacin or 40% millet enhanced both growth and the protein efficiency ratio, an experiment was devised to determine whether the supplementary effect of millet was due primarily to its niacin. The millet contained 1.40 mg% of niacin. Groups of rats were fed the basal diet + 40% maize supplemented with different levels of millet (5%, 10%, 20% and 40%) for an experimental period of 26 days. The growth of these animals was compared to that of groups receiving the basal diet supplemented with amounts of niacin (0.06, 0.12, 0.24 and 0.48 mg%) equal to that in each of the millet supplements. The results are shown in table 3. The addition of various levels of millet to basal + 40% maize diet (replacing an equivalent amount of sucrose) enhanced growth above that obtained when the equivalent amount of niacin was added. The best growth was obtained with the basal + maize diet supplemented with 20% or 40% millet. The addition of niacin alone did not enhance the growth of the rats on a 40% maize diet until the level of 0.24 mg% was reached. It should again be noted that although the growth obtained with millet supplementation was superior in every case, the protein efficiency ratios were not uniformly superior.

Inasmuch as the niacin content of millet did not appear to be the sole factor responsible for the enhancement of growth of rats receiving the basal + maize diet, a study was



drift. This suggests that amino acids, other than tryptophan, provided by the millet are used less efficiently when present in higher amounts and could be due to the fact that, as the millet content of the diet is increased, lysine becomes more and more limiting. It is unlikely that the capacity of the rat to synthesize protein has been exceeded in these experiments.

TABLE 4

*Supplementary effect of millet, tryptophan and niacin-tryptophan on the growth of rats<sup>1</sup> receiving a 9% casein + 40% maize diet*

SUPPLEMENTS TO 9% CASEIN + 40% MAIZE DIET	AVERAGE GROWTH IN 27 DAYS	P.E.R.	TOTAL PROTEIN INTAKE IN 27 DAYS	NIACIN INTAKE PER 100 GM DIET	TRYPTOPHAN INTAKE PER 100 GM DIET
%	gm		gm	mg	mg
None	78 ± 8 <sup>2</sup>	2.42	32.25	1.10	133
Millet, 5	96 ± 5	2.66	38.08	1.16	144
Tryptophan, 11 mg	100 ± 3	2.51	35.59	1.10	144
Niacin, 0.06 mg + tryptophan, 11 mg	97 ± 3	2.51	38.64	1.16	144
Millet, 10	104 ± 5	2.55	40.72	1.22	155
Tryptophan, 22 mg	96 ± 1	2.64	36.35	1.10	155
Niacin, 0.12 mg + tryptophan, 22 mg	103 ± 3	2.63	39.10	1.22	155
Millet, 20	105 ± 5	2.26	46.48	1.34	177
Tryptophan, 44 mg	101 ± 6	2.67	37.76	1.10	177
Niacin, 0.24 mg + tryptophan, 44 mg	103 ± 3	2.57	40.09	1.34	177

<sup>1</sup> Seven animals were used in each group.

<sup>2</sup> Standard error of the mean.

When millet is eaten by humans it may constitute a very high percentage of the dietary. Accordingly, a further study was made of the effect of this cereal when given as the sole source of protein in the diet, i.e. without casein. The growth performance of rats fed a diet containing 89% millet for an experimental period of 21 days is given in table 5. Rats on the 89% millet diet grew poorly during the 21-day period with an average weight gain of but 18 gm and a protein efficiency ratio of 1.06. Supplementing the diet with niacin or tryptophan or both did not improve growth or the protein

supplementation. This finding confirms the amino acid analyses which indicated that this cereal was deficient in lysine.

When maize was the sole cereal and only source of protein in the diet, growth was also very poor and the addition of niacin or tryptophan or both had no effect. Addition of millet at the levels 2, 5 and 10%, as well as of niacin and tryptophan

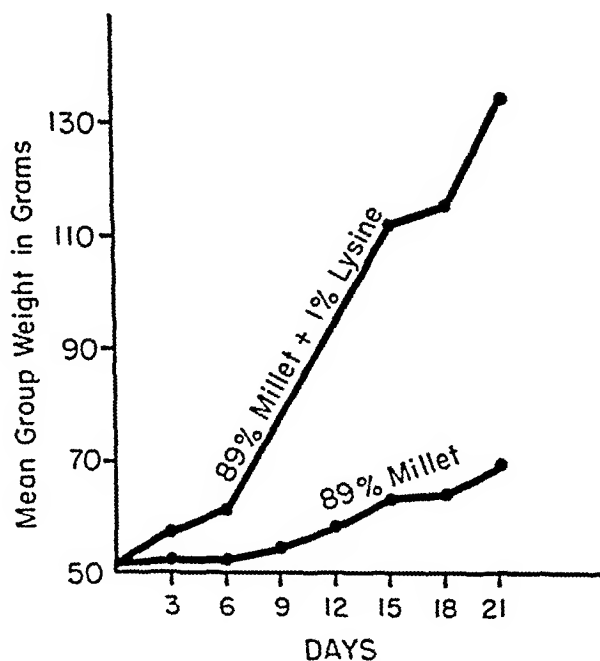


Fig. 2 Effect of lysine supplementation on the growth of rats fed an 89% millet diet.

supplements to the 80% maize diet, did not improve the growth or protein efficiency ratio. However, addition of lysine to a diet of 80% maize + 10% millet + niacin + tryptophan improved growth appreciably with a resulting total weight gain of 52 gm and protein efficiency ratio of 3.05. Lysine was without effect in the absence of niacin and tryptophan, indicating that the diet of 80% maize + 10% millet is deficient in tryptophan and lysine to about the same

It is evident that as the millet content of a maize diet is increased, the diet becomes more deficient in lysine and less so in tryptophan. This, combined with the fact that millet corrects a maize-induced niacin deficiency in the rat, seems to explain the low incidence of pellagra in those maize-consuming areas where millet is also used, and further suggests that under such circumstances one may encounter a limitation due to lysine. The improvement in growth of the rat brought about by the addition of lysine to a whole millet diet and to certain maize-millet mixtures is evident. Clinical manifestations of lysine deficiency have not been described in humans, and the requirements of man under such conditions is not known. The consequences of pellagra are well known. It would appear that in regions of endemic pellagra where the dietary is largely maize, one might beneficially encourage the use of millet. In such cases, careful attention should be given to selection of a variety of millet of high tryptophan content, such as the species here studied. Because of the variation in composition of millets, further knowledge of the amino acid composition of the various types is desirable. From the amino acid and niacin content of wheat, it should have a similar usefulness in maize diets and, again, the epidemiological observations are in accord with such expectations.

In regard to the nutritional value of millet in the human diet, it is of interest that Marie ('10) remarked on the absence of pellagra among the millet eaters of Egypt, and that Pieraerts ('42) observed a decreased incidence of kwashiorkor in the Belgian Congo in a year when both maize and millet were available for incorporation into the manioc bread consumed as the principal food.

#### SUMMARY

1. Microbiological analyses of Italian millet (*Setaria italica*) for the essential amino acids suggested that this cereal was deficient in lysine, but of high tryptophan content. The analytical data were confirmed by the findings that millet will

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# TERATOGENIC EFFECTS OF PANTOTHENIC ACID DEFICIENCY IN THE RAT

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Nelson and Evans ('46) demonstrated that pantothenic acid deficiency in the rat resulted in fetal death with resorption or in undersized young, some of which were stillborn. Since then, Lefebvres-Boisselot ('51) has observed a high incidence of cerebral and eye defects, digital hemorrhages and edema in rat fetuses from pantothenic acid-deficient mothers. Zunin and Borrone ('54) have reported the occurrence of similar anomalies in the offspring of pregnant rats given pantothenic acid-deficient diets containing the antimetabolite, pantoyltaurine. These reports have prompted our reinvestigation of the effects of pantothenic acid deficiency on embryonic development in the rat. The effects of the vitamin antimetabolite, omega-methyl-pantothenic acid, have also been studied.

## METHODS

Stock female rats of the Long-Evans strain, 60 to 65 days of age, were placed on the pantothenic acid-deficient diet on the day of breeding; others were bred after approximately 4, 10 or 20 days of the vitamin deficiency and continued on the deficient diet. Control animals received the pantothenic acid-deficient diet for 18 to 20 days before breeding and

<sup>1</sup> Presented in part at the 20th annual meeting of the American Institute of Nutrition, April, 1956 (Evans et al., '56). This research was aided by grants from the United States Public Health Service A-541 and the Roche Anniversary Foundation.

term experiments in the rat unless specially purified proteins are used or acute stress conditions imposed. Stock rats of the Long-Evans strain have been shown to have sufficient stores of vitamin B<sub>12</sub> to meet the stress of lactation on purified diets<sup>6</sup>. In studies on riboflavin deficiency (Nelson et al., '56), supplements of vitamin B<sub>12</sub> were given but it was not possible to detect any significant differences related to this vitamin.

TABLE 1

*Effect of pantothenic acid deficiency on fetal development in the rat*

DEFICIENCY PRIOR TO BREEDING	RATS BRED	WT. CHANGE DURING GESTATION	RESORP- TIONS	LITTERS	YOUNG		
					Total no.	Avg. no. per litter	Abnormal
days	no.	gm	%	%			%
Pantothenic acid-deficient diets throughout gestation							
0	23	+ 74	22	78	160	8.9	14
4	20	+ 53	32	68	87	6.7	29
8	21	+ 27	57	43	45	5.0	20
10	20	+ 29	70	30	44	6.3	16
20	21	— 6	100	0	0		
Pantothenic acid-supplemented diets throughout gestation							
19	24	+115	0	100	249	10.4	0

## RESULTS

Table 1 shows that when pantothenic acid deficiency was instituted at the beginning of pregnancy, fetal death with resorption of the entire litter occurred in 22% of the animals. In the living young, 14% showed macroscopic abnormalities. When the deficiency was started just 4 days before breeding, embryonic development was more severely affected, the incidence of abnormal young increasing to 29% and fetal death with resorption to 32%. With longer periods of deficiency before breeding, fetal death with resorption of the entire litter increased, and when the deficiency was instituted 20 days before breeding all animals resorbed. The pregnant

<sup>6</sup> Unpublished data.

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\* Unpublished data.



(25 to 27%) of the pregnancies and 15 to 24% of the living young were abnormal. Accentuation of pantothenic acid-deficiency in the rat by this antimetabolite is in agreement with the findings of other investigators, e. g. Shinazi et al. ('50).

The anomalies observed macroscopically in 102 abnormal offspring (table 3) included those defects previously reported by other investigators, namely, exencephaly, (figs. 1 and 2), hydrocephalus, anophthalmia, microphthalmia, digital hemorrhages (figs. 7 to 10) and edema. In addition, interventricular septal defects, anomalies of the aortic arch pattern,

TABLE 3

*Incidence and types of macroscopic abnormalities resulting from pantothenic acid deficiency in 102 abnormal 21-day fetuses*

	%
<sup>1</sup> Microphthalmia and anophthalmia .....	32
<sup>1</sup> Edema .....	31
Hydronephrosis and hydroureter .....	31
Clubfoot, tail defects and cleft palate .....	23
<sup>1</sup> Digital hemorrhages .....	19
<sup>1</sup> Exencephaly and hydrocephalus .....	13
Dermal defects .....	13
Cardiovascular anomalies .....	12

<sup>1</sup> Anomalies reported by other investigators.

hydronephrosis and hydroureter (figs. 5 and 6), clubfoot, tail defects, cleft palate, and dermal defects (figs. 3 and 4) were observed. Single examples of undescended testes and apparent absence of the kidneys were also found. These abnormalities were similar to those observed in studies on other teratogenic dietary deficiencies, e. g. pteroylglutamic acid and riboflavin (Nelson et al., '52, '55, '56) with the exception of the digital hemorrhages and dermal defects. According to Giroud et al. ('55), the digital hemorrhages (figs. 7 and 9) are followed by degeneration of skeletal elements and sometimes by "spontaneous amputation" of the digits so that malformed paws or ectrodactyly resulted (figs. 8 and 10).

tion has been estimated by Lefebvres-Boisselot ('55) to be approximately 9 mg per kilogram (weight of conceptus) in contrast to reported requirements of 3 to 4 mg per kilogram body weight at birth, 1 to 2 mg per kilogram at weaning, and 0.5 mg per kilogram at 10 weeks of age.

The deleterious effects of a transitory deficiency of pantothenic acid induced by the antimetabolite during the second week of pregnancy are similar to those found for a transitory deficiency of either pteroylglutamic acid or riboflavin during this period. Fetal damage produced during the critical period of embryonic differentiation and organogenesis cannot be reversed by vitamin supplementation later in pregnancy. The results demonstrate the necessity of pantothenic acid for early embryonic development in the rat.

#### SUMMARY

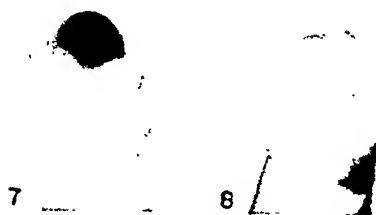
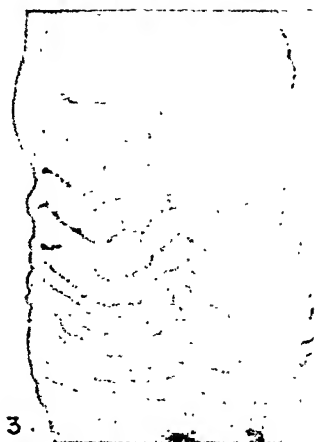
Multiple congenital anomalies in rat fetuses resulted when pantothenic acid deficiency was instituted on the first day of gestation or 4 to 10 days before breeding and continued throughout pregnancy. When the vitamin deficiency was limited to the first 12 or 14 days of the gestation period, few anomalies were observed. However, addition of the antimetabolite, omega-methyl- pantothenic acid, to the deficient diet for the last two or three days of this period accentuated the deficiency and fetal death with resorption of the entire litter resulted in some cases; in other cases more abnormal young were found than in the absence of the antimetabolite.

The abnormalities observed included those previously reported, namely, cerebral and eye defects, digital hemorrhages and edema and, in addition, interventricular septal defects, anomalies of the aortic arch pattern, hydronephrosis and hydroureter, clubfoot, tail defects, cleft palate and dermal defects.

#### ACKNOWLEDGMENTS

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PLATE



10

# THE PYRIDOXINE REQUIREMENT OF THE BABY PIG<sup>1,2</sup>

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Chick et al. ('38), Hughes and Squibb ('42), Wintrobe et al. ('43), Lehrer et al. ('51) and Moustgaard ('53) have described the various clinical and subclinical symptoms which compose a syndrome of pyridoxine deficiency in the suckling and the weanling pig. The work of Wintrobe et al. ('43) and of Moustgaard et al. ('52) indicated that 40  $\mu$ g of pyridoxine per kilogram of body weight daily is sufficient to prevent the occurrence of these symptoms in the weanling pig. However, Moustgaard ('53) found it necessary to supply twice this amount to produce maximum rates of body weight gain and protein utilization.

The present series of trials was designed to determine the pyridoxine requirement of the baby pig on a synthetic-milk diet. The purpose of this paper is to submit data which relate this requirement to the total consumption of dietary solids and also to report observations of pyridoxine deficiency symptoms that appear in pigs which are inadequately supplied with this vitamin.

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Urine collections were made weekly to determine xanthurenic acid excretion values. Collections were made over an 8- to 12-hour period. Before being placed into metabolism cages for this collection, pigs received an oral dose of DL-tryptophan amounting to 100 mg per kilogram of body weight. Urine xanthurenic acid concentration determinations were made using the method of Wachstein and Gudaitis ('52).

As in previously cited work by the present authors, gross and microscopic post mortem examinations were made on all pigs that died, those which were killed in extremis and those positive control pigs which were sacrificed to be used as comparative standards. Various organ weights were also taken and recorded. All data obtained on individual pigs throughout the experiment were grouped and statistically analyzed using the methods of Snedecor ('46) for analyzing single and multiple classification variance.

#### RESULTS AND DISCUSSION

The combined results of the three feeding trials pertaining to pig growth and feed consumption are presented in table 1. Pigs from all lots receiving pyridoxine gained significantly more rapidly and efficiently than pigs from the lot receiving no dietary pyridoxine. The daily rate of gain of pigs receiving 0.75 mg or more of vitamin B<sub>6</sub> per kilogram of dietary solids was 11 to 14% greater and their daily solids intake significantly greater than for those pigs which received 0.5 mg of vitamin B<sub>6</sub> per kilogram of solids.

The pigs which received no pyridoxine ate and gained normally for the first two weeks of experimental feeding. Then there was a gradual loss of appetite and the rate of gain dropped. Most of these pigs lost weight during the final week of the trial. A typical example of a pyridoxine-deficient pig is shown in figure 1. Vomiting with expulsion of copious amounts of a thick yellowish-green fluid occurred occasionally during the third and 4th weeks of experimental feeding. Epileptiform seizures were observed frequently in

suggested that focal cortical seizures may be due to a biochemical lesion, particularly in the metabolism of glutamic acid. Epileptiform seizures and vomiting were observed in a few of the pigs of lot 2, but none was observed in any of the pigs receiving 0.75 mg or more of vitamin B<sub>6</sub> per kilogram of solids.

A summary of the weekly blood hemoglobin determinations is made in table 2. An increasing degree of hypochromia



Fig. 1 A pyridoxine deficient pig 6 weeks of age and weighing only 8 pounds.

developed as the degree of pyridoxine deficiency advanced. This was evident in all pigs receiving 0.5 mg or less of pyridoxine per kilogram of dietary solids. Cartwright and Winthrope ('48) have shown that the fundamental disturbance causing the hypochromia in pyridoxine-deficient pigs is an inability to synthesize protophorphyrin.

Blood cell studies revealed the presence of pale microcytic erythrocytes from the third week until the end of the trial in pigs receiving less than 0.75 mg of vitamin B<sub>6</sub> per kilogram of solids. Anisocytosis was commonly observed in blood from the deficient pigs also. Data on erythrocyte count presented in table 3 suggest that oligocythemia may be a sig-

nificant factor in the promotion of pyridoxine-deficiency anemia. Dinning and Day ('56) have recently reported a significant increase in erythrocyte count in the pyridoxine-deficient rat.

Total leukocyte counts in pyridoxine-deficient pigs did not differ significantly from those of control pigs at any time during the trial. However, data in table 3 show a significant lymphocytopenia in the vitamin B<sub>6</sub>-deficient pigs. There was a corresponding increase in the percentage of neutrophils in the peripheral blood of these animals. Stoerk ('46), Agnew and Cook ('49) and Dinning and Day ('56) have found greatly reduced lymphocyte production in vitamin B<sub>6</sub>-deficient rats. Dougherty et al. ('44) have demonstrated the presence of antibodies in lymphocytes in mice and Stoerk and Eisen ('46), Agnew and Cook ('49), Axelrod et al. ('47) and Ludovici et al. ('51) have all observed reduced antibody response in vitamin B<sub>6</sub>-deficient rats.

Moustgaard ('53) has reported urine xanthurenic acid excretion to be a very sensitive measure of pyridoxine deficiency in the weanling pig. Data presented in table 4 shows this to be a sensitive test for dietary pyridoxine adequacy in the baby pig also. Inspection of the data indicates that the level of dietary pyridoxine adequacy lies between 0.75 and 1.0 mg of vitamin B<sub>6</sub> per kilogram of solids.

In studying the physiological effects of a nutrient deficiency, the research worker often wonders if the effects are a specific result of the nutrient deficiency or a result of the coincidentally reduced appetite. To test this in the present work, 4 pigs receiving the positive control diet were pair fed with 4 pigs receiving no pyridoxine. The results of this trial are presented in table 5 and demonstrate that the hypochromia, oligocythemia, lymphocytopenia and the improper metabolism of tryptophan observed in the deficient pigs were specific effects of pyridoxine deficiency and did not result from the ensuing anorexia. No evidence of microcytosis or anisocytosis was observed in blood from the pair-fed positive



vitamin B<sub>6</sub>-deficient pigs in his work. In the present study the gamma globulin fraction was found to be increased. The alpha globulin fraction was also increased and the albumin fraction was decreased in the serum of pigs receiving no pyridoxine in the diet. A summary of these data is presented in table 6. The amount of total serum protein was not significantly altered by the pyridoxine deficiency in this study.

TABLE 6

*Total serum protein concentration and electrophoretic distribution of serum proteins in baby pigs receiving different dietary levels of pyridoxine*

	LEVEL OF PYRIDOXINE IN DIET, IN MG/EG SOLIDS				
	0	0.5	0.75	1.0	2.0
Number of pigs	5	11	11	8	5
Total serum protein, gm/100 ml	5.75 ± 0.26	5.80 ± 0.18	5.60 ± 0.30	5.55 ± 0.22	5.64 ± 0.45
Serum protein distribution, % of total					
Albumin	32.9 ± 2.3	44.7 ± 0.9**	44.6 ± 1.0**	44.4 ± 2.1**	42.0 ± 1.7**
α Globulin	34.7 ± 1.7	27.8 ± 1.1**	25.4 ± 0.9**	27.2 ± 1.5**	27.9 ± 0.6**
β Globulin	16.8 ± 0.3	18.9 ± 0.8	20.9 ± 0.8*	18.8 ± 0.7	20.3 ± 1.5*
γ Globulin	15.3 ± 1.1	8.6 ± 0.8**	9.1 ± 0.4**	9.6 ± 1.2**	9.7 ± 0.8**

\* Significantly different from sera of pigs receiving no pyridoxine ( $P = 0.05$ );  
 \*\* ( $P = 0.01$ ).

The presence of a generalized subcutaneous edema in all deficient pigs on which post mortem studies were made was probably a manifestation of the low serum albumin values present in these animals. Relative values of the serum protein fractions and the values for serum total protein obtained in this study in pigs receiving pyridoxine were quite similar to those obtained by Foster et al. ('51) for fibrinogen-free plasma from pigs at weaning age.

The only consistent gross finding in post mortem studies of the deficient pig was the previously mentioned anasarca.

doxine intake. In addition, one of these pigs received a single 50 mg intraperitoneal injection of pyridoxine hydrochloride. Within a few days the anorexia subsided and normal gains followed in both pigs. By the end of the 24-day recovery period both pigs were making excellent gains. Neither of the pigs had a recurrence of the epileptiform seizures after treatment commenced. Blood components quickly returned to normal and urine xanthurenic acid excretion dropped sharply. One of the pigs was sacrificed at the end of the recovery period and post mortem examination revealed no abnormalities.

TABLE 7

*Relation of certain critical organ weights to total body weight in normal, pyridoxine-deficient and pair-fed control baby pigs*

	CONTROLS	PYRIDOXINE-DEFICIENT	PAIR-FED CONTROLS
Number of pigs sacrificed	4	9	3
<i>Relative organ weights<sup>1</sup></i>			
Heart	0.52 $\pm$ 0.01	0.63 $\pm$ 0.07	0.59 $\pm$ 0.04
Liver	2.90 $\pm$ 0.10	3.95 $\pm$ 0.50	2.72 $\pm$ 0.23
Kidneys	0.62 $\pm$ 0.02	1.07 $\pm$ 0.12**	0.56 $\pm$ 0.02
Thyroid	0.009 $\pm$ 0.001	0.016 $\pm$ 0.002	0.009 $\pm$ 0.002
Adrenals	0.016 $\pm$ 0.001	0.035 $\pm$ 0.005*	0.015 $\pm$ 0.002

<sup>1</sup> Per cent of total body weight.

\* Significantly greater than corresponding positive control relative organ weight ( $P = 0.05$ ); \*\* ( $P = 0.01$ ).

#### SUMMARY

Levels of pyridoxine supplementation constituting 0, 0.5, 0.75, 1.0, and 2.0 mg per kilogram of solids in a synthetic milk diet were used to determine the requirement of the pig for this vitamin. An analysis of the growth and feed consumption data indicates that the 0.5 mg level is nearly adequate. However, data obtained on blood hemoglobin, red blood cell and lymphocyte counts indicate that the minimum requirement is not less than 0.75 mg of pyridoxine per kilogram of solids. With respect to urinary xanthurenic acid, however, the level of 0.75 mg of pyridoxine is inadequate. It

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# THE EFFECTS OF DIFFERENT FOOD FATS ON SERUM CHOLESTEROL CONCENTRATION IN MAN<sup>1</sup>

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Though a simple reduction in the fat content of the average American diet usually produces a prompt fall in the serum cholesterol concentration (Keys et al., '50; Mellinkoff et al., '50; Stark, '50; Groen et al., '52; Mayer et al., '54; Keys et al., '55), synthetic or artificial diets containing large amounts of some vegetable oils may also depress the serum cholesterol, at least in short-time experiments (Kinsell et al., '52; Ahrens et al., '54; Bronte-Stewart et al., '56). Controlled comparisons of different food fats in normal human diets of ordinary foodstuffs have been notably lacking and the present paper reports such comparisons involving butterfat, corn (maize) oil, cottonseed oil, coconut oil, olive oil, sunflower seed oil and sardine oil fed at levels representing 30 to 35% of the total calories. In addition, the effects of these fats are compared with those of ordinary American and with low-fat diets in which fats contribute, respectively, around 40 and 9 to 16% of total calories.

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before the start of each experiment proper and the size of the food servings for each man was adjusted accordingly. Nude body weights were recorded at least every week and these measurements were the basis for further adjustments in the calorie values of the diets.

In the few instances when men became uncooperative or ill they were dropped from the test group and all data on these individuals were excluded from subsequent analysis. Weight change of as much as 2 kg in a month or 3 kg over an entire experiment was also ground for excluding men, and their data, from further consideration. For these several reasons we excluded a total of 27 out of 159 men in the entire series of experiments, 10 of the exclusions occurring in the first experiment (C) of the series. Improved selection and management of the men resulted in losing only two out of 26 men in both experiments H and J; this probably is close to an irreducible minimum for experiments averaging 5 to 6 months each in duration, including before and after controls.

*The diets.* The standard Hastings State Hospital diet, planned and prepared under the supervision of competent dietitians, conforms to the ordinary American pattern and provides an abundance of meat, milk, butter, vegetables and fruits. As served in measured portions in the Metabolic Unit, this is termed the "House Diet" and provides about 90 gm of proteins and 140 gm of fats (one-third being butterfat) per 3200 Cal. The cholesterol content of this standard "House Diet" varies from 600 to 800 mg daily, the general average of other nutrients being approximately: vitamin A, 6,000 to 10,000 I.U.; thiamine, 2.2 mg; riboflavin, 2.3 mg; niacin, 22 mg; pyridoxine, 2.2 mg; inositol, 900 mg; choline, 2500 mg; ascorbic acid, 100 to 170 mg; tocopherols, 18 mg; phytosterols, 90 mg; iron, 12 mg; calcium, 1200 mg; poly-unsaturated fatty acid glycerides, 10 gm. of which about 8 gm represents linoleic acid glycerides.

The men were maintained on this "House Diet" for at least 4 weeks before the start of each experiment with the various experimental diets. The basis of all of the experi-

portions served to satisfy the wants of the men and to maintain weight equilibrium. Plate waste was recorded on 4 representative days of each dietary period (three days in experiment J) and the average food consumption computed for these days.

*Fats in the diets.* Characteristics of the fats in the diets are summarized in table 2. These fats and oils were purchased in large lots, to ensure uniformity throughout each experiment, and were considered to be typical of good commercial

TABLE 2

*Characteristics of the fats and oils used in the present experiments*

(The iodine values are those found in the fats as used. The fatty acid values are computed from the data given<sup>1</sup> for samples of those fats having comparable iodine values. For the "House Diet" fat, fatty acid composition was computed from values for fats making up about 95% of the total fat.)

FAT	IODINE VALUE	% OF FATTY ACIDS		
		Saturated	Mono-ethenoid	Poly-ethenoid
Butterfat	32	57	39	4
Olive oil	85	12	80	8
Cottonseed oil <sup>2</sup>	108	25	25	50
Corn oil <sup>3</sup>	120	12	37	51
Sunflower seed oil <sup>4</sup>	131	10	28	62
Coconut oil <sup>5</sup>	3	97	3	0
Sardine oil <sup>6</sup>	188	23	23	54
"House Diet" fat <sup>7</sup>	50	48	45	7

<sup>1</sup> Fatty acid analyses were computed from data given by A. E. Bailey, *Industrial Oil and Fat Products*, Interscience, New York, 1951; E. W. Eckey, *Industrial Fats and Oils*, Reinhold, New York, 1954; and T. P. Hilditch, *The Chemical Constitution of Natural Fats*, 3rd Ed. Wiley, New York, 1956.

<sup>2</sup> Wesson brand.

<sup>3</sup> Mazola brand.

<sup>4</sup> Saflor brand, Co-op Vegetable Oil, Altona, Manitoba, Canada.

<sup>5</sup> The coconut oil used here was "Hydrol," a hydrogenated product of Durkee's Famous Foods, Inc.

<sup>6</sup> From Van Camp Sea Food Co., Terminal Island, California. Composition estimated from data on the degree of unsaturation of the various fractions (see Hilditch, '56).

<sup>7</sup> The iodine value computed from this fatty acid composition agrees with the tabulated iodine value which was measured directly on the mixed fat extracted from the entire diet.

was no significant difference between the effects of olive oil and those of cottonseed oil but the low-fat diet was more effective than the other experimental diets in lowering the serum cholesterol concentration from the "House Diet" level, particularly in the second low-fat diet period. Unfortunately, there was poor agreement between the two low-fat periods and the variability of the individuals was great. Nevertheless, by using all the observed cholesterol values on olive oil and cottonseed oil and comparing them with the values on the

TABLE 3

*Experiment C.<sup>1</sup> Mean serum total cholesterol concentrations at the ends of the experimental diet periods, together with the average fat intakes*

DIET	FAT INTAKE		CHOLESTEROL
	gm/day	% Cal.	mg %
"House," mean	144.6	40.9	229.4 $\pm$ 9.8 <sup>2</sup>
Low-fat, mean	28.4	9.5	198.2 $\pm$ 7.0
Olive oil	80.7	25.2	213.6 $\pm$ 9.1
Cottonseed oil	79.6	25.1	208.8 $\pm$ 10.2

<sup>1</sup> The 16 men were divided into two groups (A and B) of 8 each. Dietary periods were 4 weeks and the sequence was for group A: "House Diet," low-fat, olive oil, cottonseed oil, low-fat and "House Diet," and for group B: "House Diet," low-fat, cottonseed, olive, low-fat and "House Diet."

<sup>2</sup> Mean and standard error of the mean.

two low-fat diets statistical analysis of the individual differences indicated that the cholesterol concentration on the oil diets was significantly higher than that in the low-fat periods at the 0.05 probability level. The experience of experiment C enabled us to achieve much better control and standardization of the men and their diets in the subsequent experiments (F, H and J).

Final computation of the calorie values of the foods actually eaten on the several diets showed average values of 2883 and 2862 Cal. on the olive and cottonseed oil diets and a lower average of 2661 Cal. on the low-fat diet. The average body weight change during 4 weeks on the olive oil diet was  $\pm$  0.4 kg and on the cottonseed oil diet it was  $-$  0.2 kg; during the

corn oil diet produced an even greater decrease. Further it is apparent that the values on corn oil are significantly lower than those on both the low-fat and the cottonseed oil diets. Finally, the cholesterol values on the hydrogenated coconut oil diet were significantly higher than those on the "House Diet."

*Experiment J, including sunflower and sardine oils.* The design of experiment J is shown in table 9 together with the

TABLE 9  
*Experiment J design*

(Groups W and X contained 7 men each; groups Y and Z, 6 men each. Each dietary period consisted of two weeks and the parentheses in the table enclose the average values, in grams per day, of total fats, and the part of the fats composed of polyethenoid fatty acids for the several dietary periods. The last line gives the average daily calorie consumption for the entire experimental period for each of the 4 groups.)

PERIOD	GROUP			
	W	X	Y	Z
1	"House" (156, 11)	"House" (159, 11)	"House" (151, 10)	"House" (153, 10)
2	Low-fat (39, 3)	Low-fat (39, 3)	Butter (130, 6)	Butter (132, 6)
3	Butter (138, 6)	Butter (136, 6)	Butter (122, 6)	Butter (127, 6)
4	Olive (140, 10)	Corn (139, 58)	Olive (132, 10)	Cottonseed (128, 49)
5	Corn (140, 59)	Olive (139, 10)	Cottonseed (128, 50)	Olive (127, 10)
6	Corn (141, 59)	Sunflower (139, 10)	Corn (128, 56)	Sardine (134, 55)
7	Sunflower (141, 64)	Corn (135, 56)	Sardine (126, 52)	Corn (131, 54)
8	Butter (142, 6)	Butter (138, 6)	Butter (130, 5)	Butter (127, 5)
9	"House" (156, 11)	"House" (159, 11)	"House" (151, 10)	"House" (153, 10)
All periods	3310 Cal.	3370 Cal.	2920 Cal.	3050 Cal.



of the other diets. Note that two months between period 2 and period 3 were occupied by subsistence on constant diets but were complicated by pharmacological trials (entirely negative in effect) which accounts for the long interval between the early periods.

The sunflower seed oil diet was tested with groups W and X whereas tests with the cottonseed oil and sardine oil diets were made with groups Y and Z, so the question arises about

TABLE 11

*Serum cholesterol differences in experiment J*

(Experimental variable approximately 100 gm of fat. Cholesterol difference (mg/100 ml) computed by subtracting the value on the second diet from that on the first. Group WX included 14 men and group YZ 12 men.)

DIET COMPARISON		CHOLESTEROL DIFFERENCES (MG/100 ML) $\pm$ S.E.	
		Group WX	Group YZ
Butterfat	minus Olive	+ 35.6 $\pm$ 4.7	+ 29.3 $\pm$ 4.3
Butterfat	minus Corn	+ 62.8 $\pm$ 3.5	+ 59.5 $\pm$ 5.7
<sup>1</sup> Olive	minus Corn	+ 27.2 $\pm$ 3.6	...
Cottonseed	minus Corn	..	+ 24.0 $\pm$ 5.6
<sup>1</sup> Sardine	minus Corn	...	+ 20.1 $\pm$ 3.8
<sup>1</sup> Sunflower	minus Corn	+ 9.3 $\pm$ 2.8	...
Butterfat	minus House	+ 3.6 $\pm$ 3.8	— 0.9 $\pm$ 4.4
Butterfat	minus Low-fat	+ 38.8 $\pm$ 6.1	...
<sup>1</sup> Olive	minus Sardine	...	+ 10.1 $\pm$ 4.6
<sup>1</sup> Olive	minus Cottonseed	...	+ 6.2 $\pm$ 5.2

<sup>1</sup> Crossover experiments where half the men were maintained on one diet in parallel with the other half of the men on the comparison diet and the diets were reversed in the subsequent period.

quantitative cross comparisons between these two sets of groups. This is answered by observing the differences in groups W and X vs. Y and Z between the cholesterol values on the butterfat diet (means of periods 3 and 8) and the values on the other diets common to all groups, that is the olive oil and corn oil diets. These comparisons are given in the first two lines of table 11. Groups W, X and Y, Z, exhibited reasonable consistency in their responses to the same dietary differences.

of men. Finally, the corn oil diet was found in a class by itself yielding the lowest serum cholesterol value of all the diets.

*Beta lipoprotein cholesterol changes.* Normally, about three-fourths of the total serum cholesterol is found in the beta lipoprotein fraction. In these dietary experiments changes in the beta lipoprotein cholesterol were parallel to those in the total serum cholesterol in every dietary change. As is shown in table 12 the changes in the cholesterol in the beta lipoprotein fraction appear to account for the total cholesterol change.

In every case the change in blood serum cholesterol produced by changing the dietary fat was almost entirely accounted for by the change in the beta lipoprotein fraction.

#### DISCUSSION

The present results confirm and extend many reports that the fat content of the diet plays a central role in the regulation of the serum cholesterol level in man. It is now well known that a sharp reduction in the fat content of an ordinary American diet generally produces a prompt fall in the serum cholesterol concentration. In America, of course, a low-fat diet means primarily a diet reduced in meat fats and butterfat, since these relatively saturated food fats are most abundant in our ordinary diet. Under these conditions the cholesterol response tends to be more or less directly proportional to the degree of fat restriction so that on diets extremely low in fat, such as the rice-fruit diet, the fall in two or three weeks may amount to 30% or more of the cholesterol level on the unrestricted diet (Keys et al., '50).

The first experiment in the present series (experiment "C," started in 1952), demonstrated the serum cholesterol decrease on a low-fat diet and, further, showed that the isocaloric substitution of 50 gm daily of either olive oil or cottonseed oil for carbohydrate in such a low-fat diet produces an increase in the serum cholesterol concentration. However, this increase did not bring the level up to that characteristic of subsistence

It now appears that much of the effect of corn oil cannot be explained on the basis of these theories about the effects of the fatty acids. We have shown that change from a corn oil diet to sunflower seed oil, which is still more unsaturated and richer in linoleic acid (see table 2), produces a *rise* in serum cholesterol in man and a still greater rise results from changing from corn oil to sardine oil with an iodine number of 188 (Keys, Anderson and Grande, '57). The peculiarity of corn oil is evident in the present results in experiments H and J.

In cholesterol-fed rabbits, corn oil depresses the serum cholesterol more than a "synthetic corn oil" of the same fatty acid composition and crude corn germ has a much greater effect (Jones et al., '56). However, the extrapolation of these results to man is dangerous in view of the obviously great differences between species in cholesterol metabolism. In rats, the effects of different fats on the serum cholesterol level seem to be very different from anything yet suggested about man (Swell et al., '55; Grunbaum et al., '57).

It seems probable that at least three factors are involved in the cholesterol responses to the diets in these several sets of experiments: saturated fatty acids, di-ethenoid fatty acids, and factor "X" in corn germ and crude corn oil. Nothing has been said so far about the mono-ethenoid fatty acids, particularly oleic acid which is perhaps the most abundant of all fatty acids in the foods of the world, and it cannot be guaranteed from present information that these are completely without effect. Finally, it is questionable whether chain length of the fatty acids and the phosphatides in food fats can be ignored entirely.

Comparisons of the present findings with other results reported in the literature on serum cholesterol and the diet in experiments on man is difficult in most cases because of differences in the diet other than in fats, differences in total fat level or lack of detailed specifications as to kinds of fats in the diet. It should be emphasized especially that the present experiments involved diets of ordinary foods prepared by

observed in those experiments and, in any case, the protein intake in the present experiments was constant in the range of about 90 to 100 gm daily.

The carbohydrate intake was constant in amount and kind, within each of the experiments reported here in the comparisons between the various fats. However, the low-fat diets were, necessarily, higher in carbohydrate than the other diets. Considering this point only it would be possible to suggest that the low cholesterol values obtained on the low-fat diets may reflect either the removal of the cholesterol-increasing action of the fats or the addition of a cholesterol-depressing action of carbohydrate. The point may be academic since we are concerned here with the situation in calorie equilibrium and it is not possible to maintain this when the fat level is changed without making a corresponding change in the opposite direction in the carbohydrate intake.

Some of the most striking results in the literature are the cholesterol decreases obtained with corn oil but the diets were of the formula type and the oil was given in extremely large amounts (Kinsell et al., '52, '53, '54). In monkeys, on the other hand, a diet containing 45% of calories as corn oil produced a higher serum cholesterol level than the same diet with 10% of calories as corn oil (Portman, Stare and Bruno, '56).

Mayer et al. ('54) observed *increases* in serum cholesterol in human subjects when 70 gm of peanut oil, corn oil or vegetable oil margarine were added daily for one week to an ordinary American type diet, but no differentiation was made between these vegetable fats. Subsequently they reported a cholesterol *fall* on a mixture of corn oil and vegetable margarine (Beveridge et al., '55). The same group has recently demonstrated a major difference in effect on serum cholesterol between corn oil, on the one hand, and various animal fats on the other, when the experimental fat comprised 60% of the total calories (Beveridge, Connell and Mayer, '56). The greatest cholesterol increasing effect was observed on butterfat and further, they observed that the addition of

U. S. diet) level. Fifty grams daily of olive oil or cottonseed oil introduced into the low-fat diets also produced a significant fall but less marked than the low-fat diet alone.

4. At 50 gm daily of experimental fat, there was no significant difference between olive oil and cottonseed oil in the cholesterol response. Cottonseed oil at 100 gm daily produced a slightly lower cholesterol concentration than did olive oil fed at the same level. The basic low-fat diet also tended to produce a lower cholesterol concentration than 100 gm of olive oil daily. A much more marked depression of serum cholesterol concentration was produced by 100 gm daily of corn oil. Sunflower seed oil and sardine oil also produced significant cholesterol concentration depressions but were less effective than corn oil. Coconut oil, 100 gm daily, produced no significant change in the serum cholesterol compared with the control diet with the same total fat supplied in mixed form. The serum cholesterol values on 100 gm of butterfat daily were somewhat higher than on the control diet equal in total fat content.

5. The changes in the total cholesterol concentration were accounted for, within the limits of error, by the cholesterol in the beta lipoprotein fraction.

6. The serum cholesterol responses to the various fats corresponded roughly to the principle that saturated fats promote higher cholesterol levels than polyunsaturated fats but neither degree of saturation (iodine value) nor content of linoleic acid fully explained the results. Coconut oil is less cholesterol-promoting than would be predicted from the theories that degree of saturation or the content of essential fatty acids is the controlling factor. Sardine oil is considerably less cholesterol-depressing than would be expected if degree of unsaturation is the major factor. And corn oil caused greater depression of serum cholesterol than would be expected from either the essential fatty acid or the degree of unsaturation theories.

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# THE INFLUENCE OF COOKED VS. RAW MAIZE ON THE GROWTH OF RATS RECEIVING A 9% CASEIN RATION<sup>1</sup>

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## INTRODUCTION

Even though pellagra has ceased to be a serious problem in the United States, it is still endemic in certain maize-consuming countries of the world such as Egypt and Yugoslavia. Although the primary cause of pellagra is niacin deficiency the pathogenesis is not simple. For example, cereal diets based largely on rice may contain considerably less niacin than do many maize diets but their consumption does not produce pellagra. Of particular interest is the fact that in Mexico and in the Central American countries where maize is consumed largely in the form of tortilla, there is a low incidence of pellagra. Whether this is due to the coincident ingestion of other foods of relatively high biological value or to some nutritional improvement of the maize during preparation of tortilla has not been definitely established. In the only human feeding trials brought to bear on this problem to date, Goldsmith et al. ('56) reported that women developed niacin deficiency whether whole maize or lime-treated maize was consumed. Unfortunately, in these diets only about 20%

<sup>1</sup> This study was supported in part by grants from the Williams-Waterman Fund for the Combat of Dietary Diseases and from the E. I. du Pont de Nemours and Company.

stantiates the findings of other workers in that lime-treated maize was found to be superior to raw maize as a supplement to a 9% casein diet in the rat. However, data are also presented showing that boiling maize without the addition of lime appreciably enhances its nutritive value. Data concerning amino acid and niacin analyses of the maize products are presented and are related to the current theories regarding the pellagragenic action of maize.

#### EXPERIMENTAL

Female albino Sprague-Dawley weanling rats were placed singly at random into metal screen-bottom cages, divided into groups of various sizes depending upon the particular experiment, and fed a diet of the following basal composition: sucrose 81.8%; cottonseed oil, 5%; casein, 9%; HMW salts,<sup>2</sup> 4.0%; L-cystine, 0.2%. This was supplemented by the following amounts of vitamins in milligrams per 100 gm of diet: thiamine hydrochloride, 0.2; riboflavin, 0.3; pyridoxine, 0.25; Ca pantothenate, 2.0; choline chloride, 100; inositol, 10; biotin, 0.01; and pteroylglutamic acid, 0.02. Halibut liver oil, fortified with vitamins E and K and diluted with maize oil was added to the daily ration once each week to provide the following daily intakes; vitamin A, 400 I.U.; vitamin D, 4 I.U.; 2-methyl-1, 4-naphthoquinone, 0.06 mg, and alpha-tocopherol, 0.7 mg.

Dietary supplements were given as indicated in table 1. All supplementation of the basal diet was made at the expense of sucrose.

Maize was treated with lime by simmering 2 kg of the whole grain in 4 liters of 1% CaO until the maize was considered soft enough for grinding. This required 3 to 4 hours, a period of time in excess of that used by Cravioto et al. ('45). The mixture was then cooled, adjusted to pH 7.0 with concentrated  $H_3PO_4$ , the supernatant liquid decanted, and the

<sup>2</sup> Hubbell, R. B., L. B. Mendel and A. J. Wakeman, *J. Nutrition*, 14: 273, 1937.



perimental diets. The collected supernatant was similarly dried and ground to a powder and is referred to as "dried" extract in table 1.

Boiled maize was prepared in the same manner as lime-treated maize except that it was simmered for 3 to 4 hours in tap water. After cooling, a neutral mixture of CaO and  $H_3PO_4$  was added. The wet maize was dried and ground as indicated for the lime-treated maize.

The niacin and tryptophan contents of raw maize, boiled maize, limed maize, and limed-maize extract, were determined microbiologically using *L. arabinosus* as the assay organism. For "total" niacin assay 0.5 gm of each sample was hydrolyzed for 30 minutes with 1 N  $H_2SO_4$ . Water extracts of the maize products were prepared by autoclaving 0.5 gm in distilled water for 30 minutes or by extracting it by stirring for half an hour in a large volume of water at room temperature. The methods used in the paper chromatographic studies of niacin will be described in conjunction with the results of the studies. Samples for assay of "total" tryptophan were hydrolyzed according to the method of Greene and Black ('44) and extracts were prepared for assay of "free" tryptophan by hot water extraction as noted above. Assays were read either turbidimetrically or titrimetrically depending on the particular experiment. The two methods of measurement gave comparable results.

Commercially prepared assay media were employed for the determination of leucine, methionine, lysine, isoleucine, cystine, and phenylalanine using *Leuconostoc mesenteroides* ATCC 18042 as the test organism. *Streptococcus faecalis* 29-21, and *Lactobacillus arabinosus* ATCC 8014 were used for the assay of threonine and histidine respectively according to the procedure of Stokes et al. ('45).

The maize samples were prepared for amino acid assay as follows:  $\pm 0.5$  gm of sample was accurately weighed and placed into a small ampule with 5 ml of 10% HCl. The ampule was then evacuated by use of a water pump and sealed in a flame. After autoclaving for 16 hours at 15 lbs. pressure

somewhat better growth, both boiled maize and limed maize have identical protein efficiency ratios. This indicates that boiling *per se* is sufficient to enhance the biological value of maize and that the apparent role of lime has been overemphasized through failure to employ cooked rather than raw maize as the standard of reference.

A dried extract of limed maize was fed in combination with limed maize in one experiment. There was no difference in the growth obtained when compared to that of limed maize alone. When the 9% basal diet was supplemented with this extract growth was somewhat enhanced. This was probably due to the niacin and tryptophan supplied by the extract. This evidence fails to support the hypothesis of a "toxic factor" in the limed maize. The results could be interpreted to indicate that if any toxic factor is present it is destroyed rather than extracted.

Niacin supplementation of the basal diet and of that containing raw maize enhanced both growth and protein efficiency ratios. Niacin supplementation of boiled or limed maize diets did not appreciably improve growth or protein efficiency ratios. Tryptophan supplementation enhances growth on all diets, but again protein-efficiency values were increased only on the basal and raw maize diets. In general, supplementation with both niacin and tryptophan did not improve growth or protein efficiency ratios above those obtained on either nutrient alone.

*Chromatographic studies.* The poor growth obtained with raw maize can be readily corrected with niacin or tryptophan whereas these nutrients do not appreciably improve the growth obtained with limed maize or boiled maize. For this reason it appeared as if niacin, tryptophan or both of these nutrients, might be more available from the latter products. Accordingly, microbiological analyses for niacin and tryptophan were performed.

Table 2 details the partition of total, water extractable, and non-water extractable niacin and tryptophan in the various dietary supplements studied. The total tryptophan content

It is evident from figure 1 that the water extractable niacin activity present in boiled and limed maize occurs exclusively as niacin but that a small part of the niacin activity of raw maize is present in a form that does not move from the origin of the chromatogram.

After these initial analyses had been completed the publication of Kodicek et al. ('56) came to our attention in which chromatographic evidence was presented indicating that maize

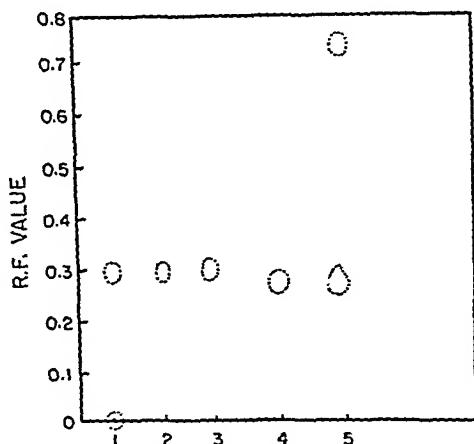


Fig. 1 Paper chromatograms of room temperature water extracts of maize, limed maize, and boiled maize. Solvent: *n*-butanol saturated with water. Areas of microbiological activity are enclosed by the broken lines. 1, raw maize; 2, limed maize; 3, boiled maize; 4, niacin; 5, niacin + niacinamide.

contained no free niacin. On this account it was deemed advisable to repeat our own work and attempt to duplicate that of Kodicek.

Accordingly, 5 gm of ground maize was refluxed for one hour on a water bath with 20 ml of 80% (v/v) methanol that was 0.1 N in HCl. After centrifugation and removal of the supernatant the residue was re-extracted once with 20 ml and twice with 10 ml of the same solvent. The combined extracts were then evaporated to half their volume (30 ml) on a steam bath. Instead of using ion-exchange resin at this point to remove HCl as was done by Kodicek et al., the evaporation

After alkaline hydrolysis (fig. 2 A, 3 and 4) the areas of microbiological activity coincided with the CNBr reactive areas. When authentic niacin was added to this mixture before chromatography no additional spots were detected. A mixture of niacin and niacinamide (fig. 2 A, 5) also gave coincident results. These findings, then, confirm those of Kodicek et al. ('56) and, in addition, demonstrate the absence

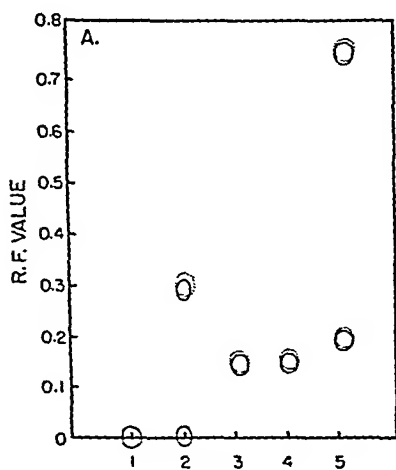


Figure 2 A

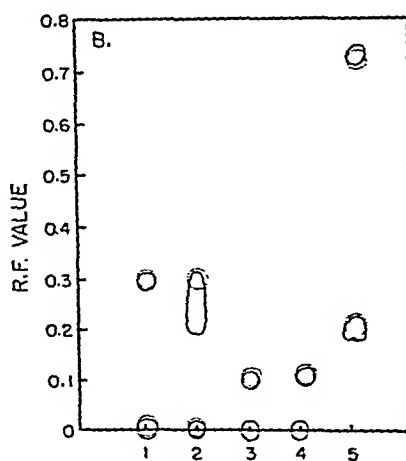


Figure 2 B

Fig. 2 Paper chromatograms of (A) 80% methanol-HCl extract of maize and (B) water extract of maize. Solvent; *n*-butanol saturated with water. Areas giving the cyanogen-bromide reaction are enclosed by solid lines. Areas of microbiological activity are enclosed by the broken lines. 1, unhydrolyzed extract; 2, unhydrolyzed extract + niacin; 3, alkali-hydrolyzed extract; 4, alkali hydrolyzed extract + niacin; 5, niacin + niacinamide, 1  $\mu$ g each.

of microbiologically available niacin in a methanol-HCl extract of maize.

The chromatographic patterns obtained using a water extract of maize contrast sharply with those obtained with the methanol-HCl extract. Figure 2 B (samples 1 and 2) shows that free niacin was detected by both the chemical and microbiological techniques. In addition, "bound" niacin was also present in the water extract and exhibited slight microbiologi-

TABLE 3

*Niacin partition in water extracts of maize determined by microbiological assay of paper chromatograms*

SAMPLE CHROMATOGRAPHED	Method of assay	NIACIN CONTENT $\mu\text{G}/0.5 \text{ ML EXTRACT}$					Pre- dicted total content (Tube assay)	% (Re- covery total)
		"Bound"	Rf	Free	Rf	% Bound		
(1) H <sub>2</sub> O Extract	Aseptic	0.201	0	0.298	0.32	...	0.515	104 <sup>1</sup>
(2) H <sub>2</sub> O Extract: NaOH treated	Aseptic	0.242	0	0.263	0.33	48	0.515	98
(3) H <sub>2</sub> O Extract	Non-aseptic	0.030	0	0.343	0.44	...	0.515	97
(4) H <sub>2</sub> O Extract: NaOH treated	Non-aseptic	0.157	0	0.374	0.37	29.6	0.515	103

<sup>1</sup> Total recovery calculated using "bound" niacin content obtained on NaOH-treated extract, and the predicted total content determined by tube assay of an NaOH-treated extract.

TABLE 4

*Niacin partition in water extracts of maize as determined by non-aseptic microbiological assay*

SAMPLE ASSAYED	NIACIN CONTENT			
	(A) No treatment	(B) Hydrolysis <sup>1</sup> with NaOH or H <sub>2</sub> SO <sub>4</sub>	(B-A) Bound niacin	Bound niacin
	$\mu\text{g}/\text{gm}$	$\mu\text{g}/\text{gm}$	$\mu\text{g}/\text{gm}$	%
H <sub>2</sub> O extract of maize: ½ hour at room temperature	6.76	18.36	11.60	63.2
H <sub>2</sub> O extract of maize: 10 hours at room temperature	14.22	20.33	6.11	26.2
H <sub>2</sub> O extract of maize: Soxhlet extracted for 15 hours	28.62	29.07	0.45	1.5

<sup>1</sup> The value for the acid and alkaline hydrolyzate were so similar that they are presented here as an average.

The most logical explanation for the lack of quantitative niacin extraction is that the starchier components of the grain are easily extracted whereas the more fibrous portions of the grain and the oily germ are not. Since exhaustive Soxhlet extraction removes the niacin quantitatively it is probable that the partition of niacin in the fraction that is not extracted at room temperature is identical with that that is.

These results demonstrate that the apparent forms of niacin present in maize vary according to the method of extraction. Although our results indicate that "bound" niacin represents from 30 to 60% of the niacin activity of maize, it is probable that only "bound" niacin is present in maize — this being readily converted to the free form in aqueous solution. Additional evidence for this belief is that only "bound" niacin can be detected in methanol · HCl extracts. The rapid conversion of the "bound" to the free form in water explains our finding that boiled maize is as effective as limed maize in supporting the growth of rats. In essence, our findings lend considerable support to the claim of Kodicek et al. that the effectiveness of lime treatment of maize in curing a niacin deficiency depends upon the degree of hydrolysis of the "bound" form of niacin.

*Amino acid analyses.* Massieu et al. ('49) reported that considerable loss of amino acids occurred during the preparation of tortilla and Cravioto and co-workers ('52) suggested that this might result in an improved amino acid balance. Since imbalances of certain amino acids have been demonstrated to produce niacin deficiency in rats fed low-protein diets (Koepppe and Henderson, '55) it was considered pertinent to study this possibility.

The tryptophan loss during the cooking process proved to vary considerably from batch to batch (tables 2 and 5). The very low tryptophan content of maize contributes to this variability in that relatively small quantitative differences assume large proportions when calculated on a percentage basis. Consequently, we attach no particular significance to this lack of agreement and feel that the superiority of cooked

The large loss of threonine reported by the Mexican workers could not be confirmed in our study. However, because of the findings of Koeppe and Henderson ('55) that the addition of threonine to a low-tryptophan diet can produce a niacin deficiency in the rat, a study was made of the effects of the addition of threonine to the cooked maize diets. The results indicated that threonine was without effect which dispelled the possibility that a cooking loss of threonine might have effectively lowered the niacin requirement. It might be added that a similar study of lysine supplementation of the cooked products was also without effect. Very little hydrolysis, if any, took place during the cooking process; practically no free amino acids could be detected in the cooked products. Presumably, the small measurable quantities found in the raw maize are lost in the cooking water.

The fact that no gross changes in amino acid content were observed plus the finding that supplementation with the two amino acids deemed most likely to influence the niacin requirement was ineffectual, detracts considerably from the "amino acid imbalance concept" proposed by Cravioto et al. ('52). Although reduced digestibility of the cooked product with the preferential excretion of a grossly imbalanced protein fraction (such as zein) could conceivably improve the balance in the intact animal, satisfactory experimental evidence has not been obtained for this belief. Consequently, it is concluded that the superiority of cooked maize to raw maize in the 9% casein diet of the rat is due to the liberation of free niacin from the normally unavailable "bound" form during the cooking process. The chemical nature of this "bound" niacin has not been determined.

#### SUMMARY

Data have been presented confirming reports in the literature that limed maize permits more rapid growth of rats than does raw maize when added to a 9% casein, niacin-free diet. The finding that boiled maize was equivalent in this

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# APPLICATION OF THE PROTEIN DEPLETION- REPLETION TECHNIQUE IN BABY PIG FEEDING EXPERIMENTS

## I. A COMPARISON OF LEVELS AND SOURCES OF PROTEIN FOR BABY PIGS <sup>1,2</sup>

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The most widely used method for testing the adequacy of protein in baby pig diets has been to compare the responses of the pigs fed different kinds and amounts of protein in terms of efficiency of feed utilization, and the magnitude of weight gain during periods of uninterrupted growth. However, this method has been criticized on the grounds of sensitivity. It appeared, therefore, that there is need for a more sensitive method that would allow the detection of smaller differences in responses both between sources of protein and between levels of protein.

There are several methods that have been used for this purpose. These methods include the protein efficiency method

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shavings were used for bedding and the pens were cleaned daily. Thermostats were set to hold the temperature of the rooms at 18 and 21°C. for experiments 692 and 699, respectively.

*Rations.* In both experiments a 24% protein prestarter ration (I.S.C. Pre-starter "75," Speer et al., '54) was fed

TABLE 1  
*Composition of basal rations*

INGREDIENT	PROTEIN-FREE DEPLETION RATION	20%-PROTEIN REPLETION BASAL RATION	12%-PROTEIN REPLETION BASAL RATION
	lbs.	lbs.	lbs.
Dried skimmilk (low-heat, spray-dried)	....	58.80	35.30
Sucrose	10.00	10.00	10.00
Lactose	78.02	20.17	42.29
Lard (stabilized)	2.50	4.70	4.82
Dicalcium phosphate	4.70	1.50	2.76
Calcium carbonate	0.30	0.35	0.35
Trace mineral mixture <sup>1</sup>	1.63	1.63	1.63
Woodflock <sup>2</sup>	2.00	2.00	2.00
Iodized salt	0.50	0.50	0.50
Vitamin-antibiotic premix <sup>3</sup>	0.35	0.35	0.35

<sup>1</sup> Contributed the following minerals in p.p.m.: Fe, 362.5; Cu, 7.6; Co, 3.1; Zn, 32.4; Mn, 101.5; K, 3979.0; I, 0.4.

<sup>2</sup> Manufactured by Brown Company, 110 South Dearborn, Chicago, Illinois.

<sup>3</sup> Each pound of complete ration contained at least the following amounts of vitamins and antibiotics: vit. A, 5000 I.U.; vit. D<sub>2</sub>, 1000 I.U.; riboflavin, 5.0 mg; pantothenic acid, 10 mg; niacin, 30 mg; choline chloride, 450 mg; ascorbic acid, 300 mg;  $\alpha$ -tocopheryl acetate, 10 mg; biotin, 20.0  $\mu$ g; folic acid, 9  $\mu$ g; inositol, 250 mg; Menadione, 3.0 mg; PABA, 8.0 mg; pyridoxine HCl, 1.2 mg; thiamine-HCl, 5.0 mg; vit. B<sub>12</sub>, 20.0  $\mu$ g; chlortetracycline, 15.0 mg; oxytetracycline, 15.0 mg; penicillin, 10.0 mg; bacitracin, 10.0 mg.

to the pigs for a pre-experimental period of one week. All rations were fed in the meal form. Feed consumption was determined weekly and the feeders were meticulously cleaned between the pre-experimental, the protein-depletion and the protein-repletion periods.

In experiment 692 a single cycle of a one-week depletion and a one-week repletion was employed; in experiment 699 the pigs were carried through three such cycles in succession.

## RESULTS AND DISCUSSION

*Experiment 692*

A summary of the effect of protein depletion and protein repletion on gains and feed efficiencies of baby pigs is presented in table 2.

*Gain.* During the one-week protein-depletion period, the pigs lost approximately 0.3 to 0.5 lb. of body weight per pig. Depletion starting weight did not appear to influence the amount of weight lost during the depletion period. During

TABLE 2

*Exp. 692. Summary of the effect of protein depletion and repletion on gains and feed efficiencies of baby pigs*

ITEMS	RATION TREATMENT		
	Dried skimmilk:	50% Soybean oil meal	50% Soybean oil meal - 0.1% DL-methionine
	<i>lbs./pig</i>	<i>lbs./pig</i>	<i>lbs./pig</i>
Depletion starting weight	9.8 <sup>1</sup>	9.9	9.4
Depletion final weight	9.3	9.5	9.1
Depletion loss	0.50	0.37	0.31
Repletion final weight	14.2	12.9	12.4
Repletion gain <sup>2</sup>	4.9	3.3	3.2
Repletion feed/gain	1.51	1.84	1.69

<sup>1</sup> Values are averages of 4 pigs per treatment.

<sup>2</sup> The experimental error mean squares for repletion gain and feed/pound gain were 0.7616 and 0.1887, respectively.

the one-week repletion period, those pigs repleted with the dried skimmilk diet gained approximately 1.6 lbs. more per pig than those fed the diets in which soybean oil meal was used as the source of protein. Supplementation of the soybean oil meal diets with 0.1% of DL-methionine did not improve gains. Statistical analysis of the gain data did not reveal any significant differences with the replications and degrees of freedom available in this experiment.

*Feed per pound of gain.* As expected from the gain data, it required less feed to produce a pound of repletion gain with the dried skimmilk than with soybean oil meal ration. Supple-

pigs fed 12% protein. However, the pigs on 16% protein failed to gain as rapidly as those on 14% protein. In view of the superiority of 20% protein over 18% protein for repleting protein-depleted pigs, one would expect 16% protein to replete the pigs as well or better than the 14% protein ration. Kjeldahl nitrogen determinations on the rations left no doubt in the authors' opinion that the pigs did receive their proper rations.

Examination of the growth curves (fig. 1) and the gain data in table 3 reveal that the average initial weight of the

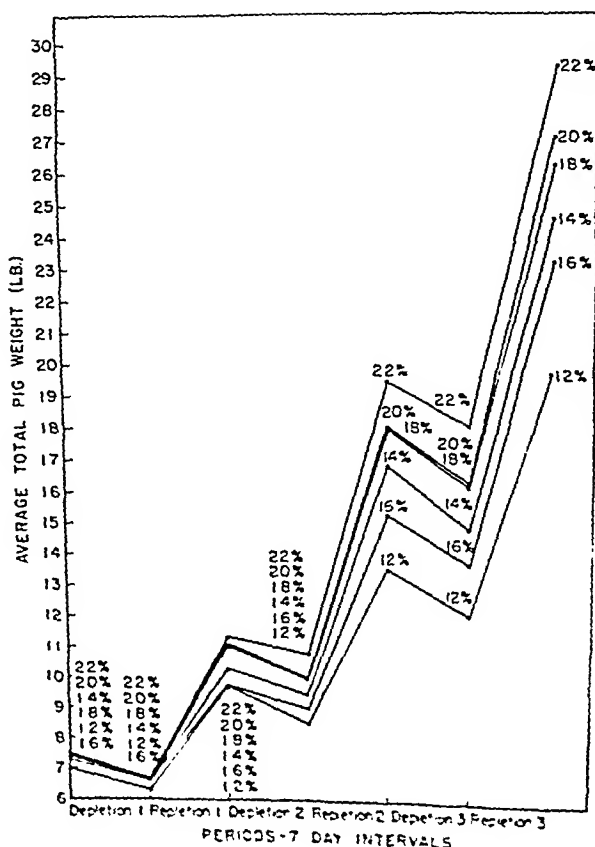


Fig. 1 Exp. 699. Growth curves of protein-depleted and protein-repleted baby pigs.

Figures in chart indicate percentage of protein in the ration. The protein was supplied by dried skim milk.

low for all levels of protein. The pigs repleted with 12% protein required the most feed, 1.71 lbs. per pound of gain. The value of 1.18 lbs. for the pigs fed 20% protein was the least amount of feed required per pound of gain. Statistical analysis of the data revealed that the linear and quadratic regression components of the protein level effects were statistically significant. This parabolic curvature in the response to protein level is largely the result of the high value of 1.71 lbs. of feed required per pound of gain for the 12% protein ration, since the values for the other levels were relatively similar.

It is realized that a direct comparison of the depletion-repletion technique with uninterrupted feeding is needed to evaluate the reliability or validity of the results obtained in this manner. However, the low variability of response obtained in these experiments as compared to similar uninterrupted feeding trials indicates that this type of experiment may prove useful in future work of this nature.

#### SUMMARY

Sixty baby pigs were used in two experiments where the protein depletion-repletion technique was employed to measure the effectiveness of three different sources and 6 different levels of protein in promoting growth.

In the first experiment, the pigs that were protein repleted with dried skimmilk diets showed greater repletion gains on less feed per pound of repletion gain than those repleted with soybean oil meal diets with or without 0.1% of DL-methionine. There was little difference in the repletion gains or in feed utilization by the pigs fed the two soybean oil meal diets. Less of the methionine supplemented ration was required to produce a pound of gain, however this difference was not statistically significant.

When 6 levels of protein were tested using dried skimmilk as the source of protein, the greatest gains were made by the pigs repleted with 22% protein whereas the least gains were made by the pigs repleted with 12% protein. Statistical

# APPLICATION OF THE PROTEIN DEPLETION- REPLETION TECHNIQUE IN BABY PIG FEEDING EXPERIMENTS

## II. EFFECT OF LEVELS OF PROTEIN ON REPLETION GAINS AND BLOOD SERUM COMPONENTS OF BABY PIGS <sup>1,2</sup>

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In the first paper of this series, it was reported by Peo et al. ('57) that the greatest repletion gains were made by pigs fed 22% protein, the highest level fed. Therefore, it would appear that this level, or perhaps even a higher level of protein, is necessary to obtain maximum repletion gains. It was the purpose of this experiment, therefore, to establish the minimum level of protein (employing dried skimmilk as the source of protein) for maximum response from protein-depleted baby pigs.

Although the repletion gain was accepted as a good criterion of response, it was considered that other criteria might be more sensitive. Thus, it was also the purpose of this experiment to determine the effect of protein depletion and repletion on the albumin/globulin ratio and on other blood components of the blood serum of baby pigs.

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of protein that could be obtained with dried skim milk as the sole source of protein and with the other ration components (and levels of each) used.

*Blood.* The pigs were bled by the heart puncture technique at the end of the preliminary, the depletion, and the repletion periods. A 5-ml syringe equipped with a number 19 needle was used to obtain a blood sample from each pig. The needles were autoclaved and the syringes were scrupulously cleaned after each bleeding period. Immediately after the blood was drawn from the pig, a portion was allocated (in a citrated tube) for hemoglobin, hematocrit and red and white blood cell determinations. The remainder of the blood was allowed to clot and then centrifuged at 1500 revolutions per minute for 15 minutes. After centrifugation, the serum was removed by pipette, transferred to a test tube and taken to the laboratory for serum protein determinations. All determinations were made within 24 hours after the blood samples were taken. The serum proteins were determined according to the method of Kingsley, described by Hawk et al. ('51). Globulin concentration was taken as the difference between the determined total serum protein and the determined albumin concentrations. Hemoglobin determinations were made according to the acid hematin method as outlined by Klett-Summerson ('47).

*Analysis of the data.* All data (gain, feed and blood) were analyzed by pooling the data for the two depletion and two repletion periods and testing according to the following analysis of variance plan.

*Analysis of Variance Plan*

<i>Source of variation</i>	<i>Degrees of freedom</i>
Replication (litters)	5
Levels of protein	5
Linear regression component	1
Quadratic regression component	1
Cubic regression component	1
Remainder	2
Remainder (Experimental error)	25
Total	35

All statements concerning statistical significance are made at a probability level of 5% or less.

that the quadratic regression component of the effects of protein levels was significant.

*Feed per pound of gain.* For the first repletion period, the feed required per pound of gain (fig. 1) decreased as protein levels were increased up to 21% protein. Except perhaps for 12% protein, the feed efficiencies for all levels of protein were exceptionally good. At the end of the second repletion period,

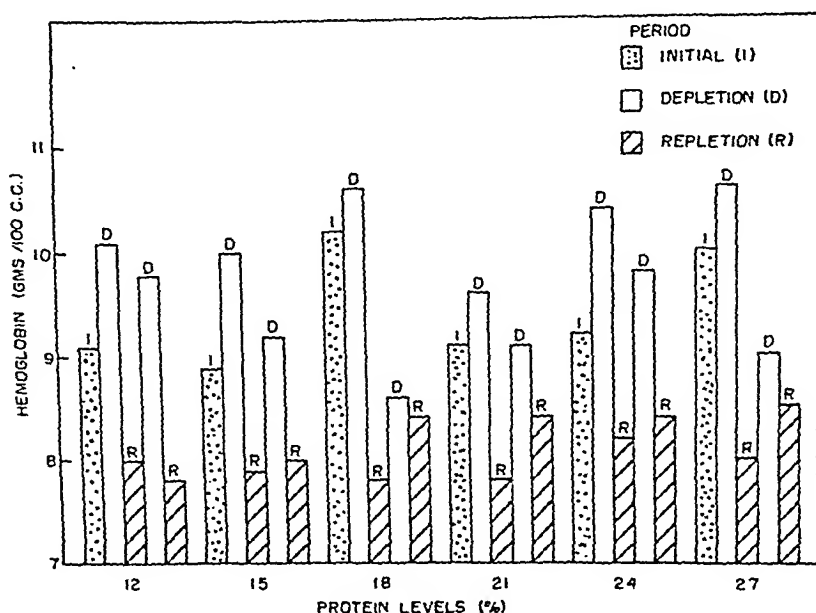


Fig. 2 Effect of protein depletion and repletion on hemoglobin levels of baby pigs.

less feed was required to produce a pound of repletion gain with 18% protein than with any of the other levels of protein. The feed data for the two repletion periods were pooled and analyzed according to the previously described analysis of variance plan. Both the linear and quadratic regression components of the treatment effect of protein levels were statistically significant with the largest portion of the mean squares for protein levels being accounted for by the quadratic component.



above 18% protein for the second repletion period. On the average, the lowest level of hemoglobin was observed in the pigs fed 12 and 15% protein during the repletion period. However, statistical analyses of the pooled data failed to reveal any significant difference in hemoglobin levels attributable to the protein levels fed during the repletion periods. This was also the case for the depletion periods and for the average differences between the two periods.

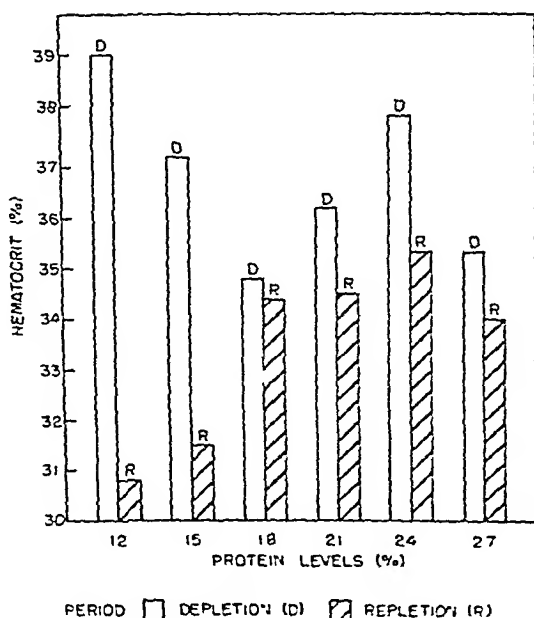


Fig. 3 Effect of protein depletion and repletion on the hematocrit of baby pigs.

*Hematocrits.* During the second protein depletion and repletion periods, hematocrit determinations were made to help clarify plasma volume changes. As shown in figure 3, the hematocrit was definitely lowered from the depletion level after the pigs were protein repleted. This indicates (although not conclusively) that plasma volume decreases during protein depletion and increases with protein repletion. Hematocrit changes were greatest on 12 and 15% protein. This indicates,

repletion red blood cell counts revealed that the linear regression component of the effect of protein levels was statistically significant. While no regression component was significant for the depletion period, the linear component was for the difference between the two periods. As the level of protein was increased the difference in the red blood cell count became less (fig. 4).

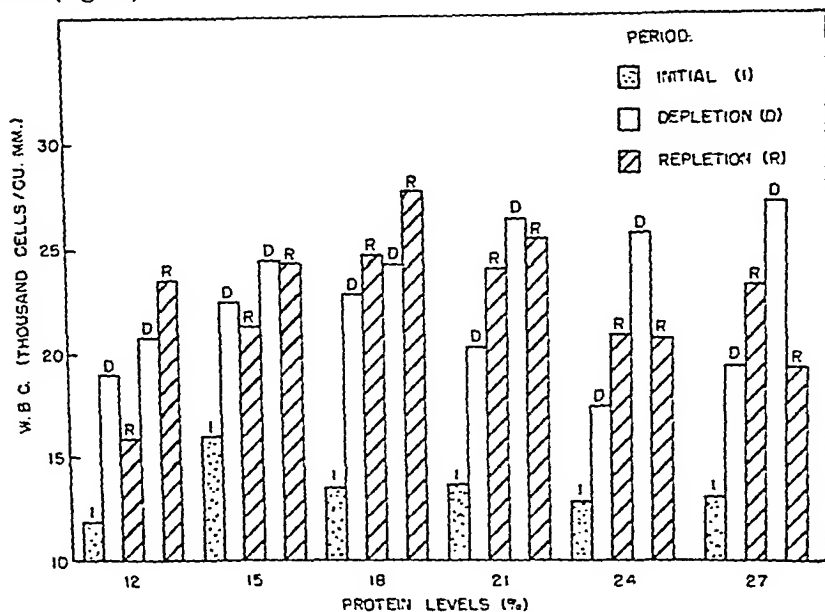


Fig. 5 Effect of protein depletion and repletion on the white blood cell count of baby pigs.

*White blood cells.* The white blood cell counts are presented in figure 5. Since white blood cells fluctuate in accordance with disease level, the observed white blood cell count does not in all instances follow the same trends as the hemoglobin level or the red blood cell count. The pigs were bled by heart puncture once each week at the experiment station farm and extreme aseptic techniques such as might be practiced in an animal clinic were not feasible. This may account for the relatively high white blood cell count observed in these pigs (Scarborough, '31, reported that on an average,

level observed at the end of the second protein-repletion period. Such a consistent decline in serum albumin was not quite as evident in the pigs repleted with higher levels of protein. The globulin fraction of the serum protein (fig. 7) did not appear to change as much as the albumin fraction. This is in agreement with the results of Zeldis et al. ('45) who showed that restriction of dietary protein results in a

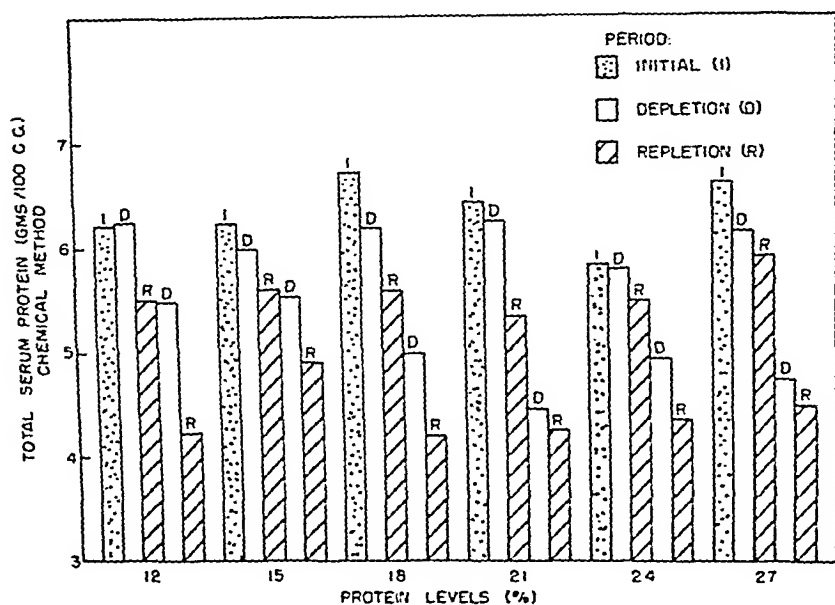


Fig. 6 Effects of protein depletion and repletion on total serum protein of baby pigs (chemical method).

decreased albumin level while globulin concentrations remain essentially normal. None of the mean squares for the regressions of either albumin or globulin approached statistical significance at the 5% probability level. The albumin/globulin ratio (fig. 7) decreased with protein depletion and increased with protein repletion. This response is in agreement with results reported by Allison ('48). If the globulin fraction remains fairly constant during protein depletion as reported by Zeldis et al. ('45), then the albumin fraction is changing

was observed with 21 and 18% protein which were also the levels that supported maximum repletion gains for the first and second repletion periods, respectively. Although the albumin/globulin ratio was greater when pigs were repleted with levels of protein higher than 12%, statistical analyses of protein level effects on the albumin/globulin ratio did not reveal this to be a significant difference.

#### SUMMARY

Thirty-six individually-fed baby pigs were used to determine the effect of 6 levels of protein (using dried skim milk as the source of protein) on the repletion gains and certain blood constituents of protein-depleted baby pigs.

Maximum repletion gains and feed utilization occurred in the pigs fed 21 and 18% protein during the first and second repletion periods, respectively. Statistical analysis showed the quadratic responses to be significant at  $P = 0.05$  or less.

The effects of protein levels on the blood components studied were probably masked by changes in plasma volume. If plasma volumes had been determined, then it is possible that the protein source and levels might have had a significant effect on blood components. Of the blood constituents studied, the albumin/globulin ratio appears to be the most promising criterion of the effects of protein depletion and repletion and warrants further investigation.

#### ACKNOWLEDGMENTS

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# THE CALCIUM, PHOSPHORUS AND MAGNESIUM BALANCES OF YOUNG COLLEGE WOMEN CONSUMING SELF-SELECTED DIETS <sup>1</sup>

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In two previous studies (Coons and Schiefelbusch, '32; McKay et al., '42) the calcium and phosphorus intakes of young college women consuming self-selected diets were determined. These intakes averaged 0.93 gm (range of 0.52 to 1.55) and 1.19 gm (range of 0.82 to 1.17) for calcium and phosphorus in the first study and 0.94 gm (range of 0.32 to 2.32) and 1.18 gm (range of 0.66 to 2.13) in the second study. Coons and Schiefelbusch ('32), using 1 gm per day as the standard intake for both calcium and phosphorus, concluded that 10 of the 17 subjects were receiving too little of both elements. McKay et al. ('42) determined the calcium and phosphorus of the feces and urine as well as the daily intake of their subjects. Although approximately 50% of their 124 subjects were in negative calcium balance on less than 1 gm intake, it was concluded that 0.8 gm could maintain calcium equilibrium in these subjects. While only slightly more than a third of these same subjects were in negative phosphorus balance on 1 gm, this amount was stated to be necessary for phosphorus equilibrium. No study of the magnesium met-

<sup>1</sup> Originated with Faculty Research Funds and financed with Williams-Waterman Grants during 1954-1956.

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Carmines was used as the fecal marker for the 5-day periods. Aliquots of both food and feces were obtained for analysis after being weighed and macerated in a Waring Blender. Aliquots were removed from the 24-hour urine collections which had been made directly into the amber gallon bottles.

The food and fecal aliquots were dried before they were analyzed gravimetrically for calcium, phosphorus and magnesium. Scott's oxalate method (Furman, '39) was used for the determination of calcium, the molybdate method (Furman, '39) for phosphorus and the ammonium phosphate method (Association of Official Agricultural Chemists, '50) was used in determining the magnesium in the filtrate from the calcium determinations. The data from these analyses were used in obtaining the total daily intakes and retentions<sup>3</sup> reported in the present study.

#### RESULTS AND DISCUSSION

The total calcium intake (food, milk, carbonated beverage and tap water) of the young Texas college women was determined for 645 days, the phosphorus intake for 500 days and the magnesium intake for 430 days. The daily urinary calcium, phosphorus and magnesium were also determined for these women while the fecal calcium, phosphorus and magnesium were determined for the 5-day period and computed to daily values in order to determine the retentions.<sup>3</sup>

The percentage of the intake absorbed<sup>4</sup> by each subject was calculated for calcium, phosphorus and magnesium to see if the subject's ability to absorb these elements might influence the retentions obtained. The average absorption values were 36% of the calcium, 69% of the phosphorus and 74% of the magnesium ingested. Since both the calcium and phosphorus percentages are higher than the usually accepted

<sup>3</sup> Calculated as 
$$\frac{\text{total intake minus fecal and urinary output}}{\text{total intake}} \times 100 = \% \text{ retained.}$$

<sup>4</sup> Calculated as 
$$\frac{\text{total intake minus fecal output}}{\text{total intake}} \times 100 = \% \text{ absorbed.}$$

TABLE 1  
*Retentions of calcium, phosphorus, and magnesium distributed for age and average total daily intake*

RANGE	CALCIUM				PHOSPHORUS				MAGNESIUM			
	Group I <sup>1</sup>		Group II <sup>2</sup>		Group I <sup>1</sup>		Group II <sup>2</sup>		Group I <sup>1</sup>		Group II <sup>2</sup>	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
	no.	no.	no.	no.	no.	no.	no.	no.	no.	no.	no.	no.
µm												
0.10-0.20									2	17	2	5
0.20-0.30										7	2	3
0.30-0.40											2	1
0.40-0.50												
0.50-0.60	3	3			2	3	1	1	2	1		1
0.60-0.70					3				3			
0.70-0.80	1	2		2					1			1
0.80-0.90		2		4								
0.90-1.00	1	2		2								
1.00-1.10	1	5		3								
1.10-1.20	2	1		4								
1.20-1.30	2	2		1								
1.30-1.40	3	4		2								
1.40-1.50	1	2		6								
1.50-1.60	2	1		4								
1.60-1.70	4	2		4								
1.70-1.80	1	1		2								
1.80-1.90	3	1		3								
1.90-2.00		2		2								
2.00-2.10	1			4								
2.10-2.20	1			5								
2.20-2.30				2								
2.30-2.40	1											
2.40-2.50	2											
2.50-2.60												
Total no.	31	32	2	28	3	11	37	25	1	25	3	14
Per cent	(57%)	(43%)	(36%)	(44%)	(83%)	(17%)	(60%)	(40%)	(40%)	(60%)	(48%)	(32%)
Average/day	1.50	1.14	1.70	1.30	1.17	0.90	1.14	1.02	0.95	0.20	1.02	0.43

<sup>1</sup>Group I composed of girls 16 to 20 years of age.

<sup>2</sup>Group II composed of girls 20 years or more of age.

group I, 24 of the 25 negative balances and 8 of the 14 in group II occurred on intakes between 0.10 and 0.30 gm magnesium. The positive balances of the two groups occurred on similar average intakes of 0.95 and 1.02 gm, respectively.

*Height in relation to calcium, phosphorus and magnesium balances.* In the two previous reports of the present long-time study (Davis and Scoular, '57; Scoular et al., '57), both height and weight were used in an attempt to obtain some common reference for comparison of the nutrient intake. In these reports the use of height as a basis for comparison of the caloric and protein intakes of the two groups was found to be better than weight although the young Texas college women were both taller and heavier than the National Research Council's 16- to 20-year olds (group I) and women 25 years of age and over (group II). Consequently, calcium, phosphorus and magnesium (all of which occur in large amounts in the skeleton) intakes are tabulated only as milligrams per centimeter of height for the subjects for the present study in table 2.

The difference between the average milligrams per centimeter intake of the subjects of groups I and II who were in positive balance was small for calcium (9.1 and 10.3 mg/cm) and for phosphorus (8.2 and 7.0 mg/cm) or negligible in the case of magnesium (6.1 and 6.4 mg/cm). The greatest differences existed between the average intakes producing positive and negative calcium balances in each group, namely 2.3 for group I and 2.4 mg/cm for group II. Seventy-eight per cent of the negative calcium balances of group I and 82% of those in group II occurred on intakes below the average intake of those having positive balances. Similarly, 91% of the negative phosphorus balances in group I and 67% in group II occurred on intakes below the average intakes of those in positive phosphorus balances. The magnesium intakes producing positive and negative balances were practically identical for the two groups (6.1 and 1.8 mg/cm and 6.4 and 2.1 mg/cm, respectively).



There were twice as many (11 as compared to 5) of the "taller" women of group I of the present study in negative calcium balance, but only two in negative phosphorus balance. The distribution of the positive and negative balances of magnesium in group I practically coincided with those for calcium. According to Stearns ('50) this may be accounted for by the fact that the solubility of magnesium is similar to that of calcium.

Group II ingested more calcium but less phosphorus than group I. Ten of the 14 negative calcium balances and 14 of the 16 negative phosphorus balances of group II occurred in the "taller" women. LeBovit and Stiebeling ('57) have suggested changes in applying the 1953 dietary allowances to U. S. population groups which include increasing the average height of women 21 to 34 years of age from 62 to 64 inches, but no change in the calcium allowance for this age group. With this adjustment the number of "taller" women who were in negative balance would be decreased to 7 in negative calcium balance and 9 in negative phosphorus balance. In group II where both the calcium and magnesium intakes were high, the calcium retentions appeared to be unrelated to the magnesium retentions.

There is no unanimity of opinion regarding the effect of the calcium-to-phosphorus ratio upon calcium retentions. In the present study, group II had the higher ratio, 1.45 and a higher percentage (63%) of positive calcium retentions than group I with a ratio of 1.2 and 40% positive retentions. Schofield and others ('56) state that the source of the calcium and phosphorus rather than the level of dietary phosphorus may influence calcium utilization in humans. They found that the same level of mineral ingestion from natural foods was better utilized than that from calcium salt. In the present study, group II consumed more milk, had higher calcium intakes and more positive retentions than group I with less milk.

The reason for the greater ingestion of milk by group II is not known since both age groups are usually included in each 5-day balance period and had access to the same food.

GROUP II (OVER 20 YEARS)

height	Calcium				Ca: P ratio	Phosphorus				Magnesium			
	Average	Intake	Balance (+)	Balance (-)		Average	Intake	Balance (+)	Balance (-)	Average	Intake	Balance (+)	Balance (-)
	gm/day	mg/cm	no.	no.	av.	gm/day	mg/cm	no.	no.	gm/day	mg/cm	no.	no.
152.6-154.9 (60-61) <sup>1</sup>	1.02 ± 0.16	6.6 ± 1.0	2	2	1.20 ± 0.14	0.87 ± 0.23	5.7 ± 1.5	2	2	0.89 ± 0.15	5.9 ± 1.0	2	2
154.9-157.4 (61-62)	1.30 ± 0.05	8.3 ± 0.2	2	2	1.38 ± 0.12	0.95 ± 0.12	6.0 ± 0.7	2	2	0.99 ± 0.36	6.3 ± 2.5	2	2
157.5-159.9 (62-63) <sup>2</sup>	1.54 ± 0.17	9.7 ± 1.1	4	2	1.67 ± 0.49	1.01 ± 0.25	6.4 ± 1.6	4	2	0.80 ± 0.30	4.9 ± 2.0	5	1
160.0-162.5 (63-64)	1.49 ± 0.52	9.3 ± 3.1	6	3	1.48 ± 0.32	0.99 ± 0.14	6.1 ± 0.9	4	5	0.82 ± 0.44	6.0 ± 3.7	3	6
162.6-165.0 (64-65) <sup>3</sup>	1.97 ± 0.33	12.6 ± 1.5	3	1	1.39 ± 0.26	1.12 ± 0.10	7.1 ± 0.6	3	1	1.49 ± 0.54	9.0 ± 3.2	4	4
165.1-167.5 (65-66)	1.61 ± 0.45	9.9 ± 2.8	3	2	1.35 ± 0.33	1.05 ± 0.19	6.4 ± 1.3	1	4	0.88 ± 0.65	5.3 ± 4.0	3	2
167.6-170.1 (66-67)	1.94 ± 0.25	11.6 ± 1.4	3	1	1.47 ± 0.28	1.06 ± 0.18	6.5 ± 1.2	2	2	1.24 ± 0.64	7.7 ± 4.0	4	4
170.2-172.6 (67-68)	2.00 ± 0.10	11.8 ± 0.6	1	1	1.74 ± 0.22	1.16 ± 0.09	6.8 ± 0.6	1	1	0.78 ± 0.52	4.6 ± 3.1	2	2
172.7-175.2 (68-69)	1.86 ± 0.29	10.7 ± 1.8	2	1	1.18 ± 0.24	1.18 ± 0.11	6.8 ± 0.4	2	1	1.18 ± 0.90	6.8 ± 5.2	2	1
175.3-177.7 (69-70)	1.67 ± 0.09	9.5 ± 0.0	1	1	1.62 ± 0.00	1.03 ± 0.00	5.9 ± 0.0	1	1	0.88 ± 0.00	5.0 ± 0.0	1	1
av. 165.0 (64.9)	1.61 ± 0.23	10.0 ± 1.4 (24)	(14)	(14)	1.45 ± 0.24	1.04 ± 0.14	6.4 ± 0.9 (22)	(16)	(16)	0.99 ± 0.45	6.2 ± 2.9 (28)	(10)	(10)

<sup>1</sup> Height in inches within parentheses.<sup>2</sup> National Research Council height for the average woman of 25 years or older.<sup>3</sup> National Research Council height for the 16- to 20-year-old girl.

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# NUTRITIONAL IMPROVEMENT OF WHITE FLOUR WITH PROTEIN AND AMINO ACID SUPPLEMENTS <sup>1</sup>

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## INTRODUCTION

Osborne and Mendel ('14) and Mitchell and Smuts ('32) showed that the proteins of wheat could be improved by the addition of lysine. Sure ('52, '53, '54) reported that the addition of threonine along with lysine to whole or milled wheat flour had a beneficial effect on growth and protein efficiency in rats.

Rosenberg, Rohdenberg and Baldini ('54) investigated the effect on the growth of rats, of adding lysine, threonine, valine and methionine to a diet in which the protein was derived from white bread which contained 3% non-fat milk solids. From their results they concluded that the only amino acid which was deficient in this commercial white bread was lysine, since no further improvement in growth was obtained by supplementing the diet with other amino acids.

However, Hundley, Ing and Krauss ('56) who explored the possibility of using algae as sources of lysine and threonine in wheat flour and bread diets, observed a growth response to

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from The Nutrition Foundation, Inc., New York, and The National Livestock and Meat Board, Chicago, Illinois. The crystalline vitamins were kindly provided by Mark and Co., Rahway, New Jersey.

At the end of two weeks the rats were sacrificed for the determination of liver fat. Each animal was stunned and decapitated and the liver was removed and stored at  $-4^{\circ}$  until the determinations were made. For the determination of liver fat, the livers were homogenized in a Potter-Elvehjem homogenizer and dried at  $100^{\circ}$ . The dried material, after grinding to a fine mesh, was extracted with ether.

The basal ration contained, in per cent, the following ingredients: white flour,<sup>2</sup> 78; corn oil, 5; salts, 4 (Hegsted, Mills, Elvehjem and Hart, '41), 4; choline chloride, 0.15; vitamin mixture, 0.25; and the various supplements of amino acids and proteins as indicated. Dextrin was added to make up to 100%. The vitamin mixture supplied in milligrams/100 gm of the ration: Thiamine HCl, 0.5; riboflavin, 0.5; niacin, 2.5; calcium pantothenate, 2.0; pyridoxine, 0.25; biotin, 0.01; folic acid, 0.02; vitamin B<sub>12</sub> 0.002 and inositol, 10.0. Fifty milligrams of vitamin C/kg of diet was added to minimize any possible destruction of thiamine (Kandutsch and Baumann, '53). Two drops of halibut liver oil, fortified with vitamins E and K and diluted with corn oil to provide vitamin A, 400 I.U.; vitamin D, 4 I.U.; vitamin E, 4.0 mg and vitamin K (2-methyl-1, 4-naphthoquinone), 0.04 mg, were given orally each week.

#### RESULTS

From the amino acid composition of white flour (Block and Bolling, '51) it is evident that when the diet contains 78% white flour as the only source of protein, all the essential amino acids except arginine, are provided at levels below the accepted requirements for the growth of the rat (Rose, '37). Since lysine was calculated to be the most limiting amino acid and was shown by Sure ('54) to give some growth response when added singly, and a correspondingly better growth response when added along with threonine to a diet containing whole wheat flour, the effect of supplementing white flour with these amino acids at various levels are recorded. The data summarized in table 1 (exp. 1) show that

<sup>2</sup> Pill-bury's enriched white flour, purchased from the open market, was used.

the addition of 0.25% of L-lysine to a diet containing 78% the flour increased the growth from 3.1 to 12.2 gm/wk. The addition of 0.2% of DL-threonine along with lysine did not further improve the growth. Increasing the level of lysine to 0.4% had no additional effect but when 0.4% of DL-threonine was included along with the higher level of lysine, the growth was increased to 21.3 gm/wk. A still better growth response (23.3 gm/wk.) was obtained when the diet was supplemented with 6% of casein.

In contrast to our previous study with polished rice (Deshmukh et al., '55) where it was observed that rats fed on a 78% rice diet developed fatty livers, which were prevented by the addition of lysine, no accumulation of liver fat was observed in any of the groups fed on the white flour, although lysine was still the limiting amino acid for growth.

In the second experiment, groups of rats were fed the white flour supplemented with amino acids or 6% of either casein or fibrin together with amino acids as indicated in Table 1. The results show that the highest levels of lysine and threonine (0.9 and 0.6% respectively) did not support growth as good as did the intermediate levels (group 10, exp. 1). A significant increase in growth was observed when certain limiting amino acids were provided with either casein or fibrin. These supplements were such that they were calculated to satisfy the requirements of the rat. When, however, a mixture of amino acids was added to meet the requirements (group 12), growth was not as good as when intact protein was included as part of the supplement. The amino acid mixture for group 12 was calculated to provide amounts equal to those provided for groups 9 and 11.

Experiments 3, 4 and 5 were included with a view to obtaining information on growth of rats when glutamic acid was added to diets containing white flour supplemented with various combinations of amino acids. It is evident that neither glutamic acid alone, nor a combination of 7 non-essential amino acids contained in 6% fibrin further improved the growth of rats fed on white flour supplemented with 9



in the presence of a lysine deficiency which depressed growth, it appeared important to investigate why no fatty livers were produced in the present experiments. The obvious difference seemed to be in the level of protein supplied by the white flour. Groups of rats were, therefore, fed on 44.2% of white flour, supplying the same protein level ( $N \times 6.25 = 5.4$ ) as provided by 90% of rice. The effects on growth and liver fat are given in table 3.

TABLE 3

*Growth and liver fat in rats fed white flour supplemented with amino acids*

GROUP NO.	COMPOSITION OF THE DIET	GROWTH	LIVER FAT
		gm/wk.	% dry wt.
1	44.2% Flour <sup>1</sup> + 0.6% DL-threonine (Basal 1)	$0.8 \pm 0.8$	$33.4 \pm 0.9$
2	Basal 1 + 0.3% L-lysine	$5.7 \pm 1.0$	$11.4 \pm 1.1$
3	Basal 1 + 0.6% L-lysine	$5.2 \pm 0.8$	$11.7 \pm 0.7$
4	Basal 1 + 0.9% L-lysine	$4.8 \pm 1.2$	$13.0 \pm 1.0$
5	44.2% Flour + 0.3% L-lysine (Basal 2)	$1.3 \pm 0.1$	$13.8 \pm 2.5$
6	Basal 2 + 0.2% DL-threonine	$10.5 \pm 1.0$	$16.1 \pm 2.3$
7	Basal 2 + 0.4% DL-threonine	$9.9 \pm 0.5$	$14.4 \pm 1.6$
8	Basal 2 + 0.6% DL-threonine	$5.7 \pm 1.0$	$11.4 \pm 1.0$

<sup>1</sup> This contained 0.11% L-lysine and 0.15% L-threonine.

It is clear that when an acute lysine deficiency was created by supplying excess threonine, rats failed to grow and at the same time fat accumulated in the liver. The addition of 0.3% of L-lysine brought the liver fat to normal but a satisfactory growth response was not obtained. Higher levels of lysine had no additional effect on growth. When, on the other hand, a threonine deficiency was created by including 0.3% of lysine in the diet, liver fat remained normal but growth was adversely affected. The addition of 0.2% of DL-threonine improved the growth from 1.3 to 10.5 gm/wk. Higher levels of threonine had a tendency to depress growth.

#### DISCUSSION

The results confirm the previous observations regarding lysine (Osborne and Mendel, '14; Mitchell and Smuts, '32)



to study the role of amino acids under a wide variety of conditions.

In the experiment cited in table 3, the higher level of threonine apparently created an imbalance resulting in a retardation of growth. The higher level of lysine was not more effective than the lower level but it did not create an imbalance. In contrast, in earlier work using rice diets (Deshpande et al., '55) an imbalance was created when the lysine level was increased whereas no imbalance was created by increasing the threonine level.

The growth of animals fed on diets containing a supplement of 6% of intact protein could be further increased by adding certain free amino acids, but 9 essential amino acids had to be added to support a rate of growth equivalent to that obtained with wheat flour supplemented with 6% of fibrin, beef or casein. Less complete combinations of amino acids tested had little effect beyond that obtained with lysine and threonine. Although several of the amino acid mixtures produced a substantial growth response, the finding that a mixture of essential amino acids simulating fibrin was not able to support as good a growth as the intact protein indicates some superiority of intact protein over amino acids as a dietary supplement under the conditions of the present experiments.

#### SUMMARY

Rate of growth of young rats was increased from 3 to 21 gm/wk when their diet, containing 78% of white flour was supplemented with 0.5% of L-lysine and 0.4% of DL-threonine. Further improvement in growth was obtained only when 7 more essential amino acids were added.

Although lysine was limiting for growth, liver fat did not accumulate when the diet contained 78% of white flour. However, fatty infiltration, which occurred when the flour was fed at a 5.4% protein level, was prevented by a lysine supplement.

Maximum growth was obtained when intact protein formed part of the supplements but growth was not as rapid when

# UTILIZATION OF AMINO ACIDS FROM FOODS BY THE RAT

## IV. TRYPTOPHAN<sup>1</sup>

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The present study of the quantitative utilization of tryptophan from foods by the rat represents a selected phase of the over-all evaluation of the nutritional quality of food proteins. Earlier work on the quantitative utilization of lysine (Schweigert and Guthneck, '53; Guthneck et al., '53) and methionine (Schweigert and Guthneck, '54) established the validity of the general approach and techniques used in this research. Further refinements of these methods have been used in the present study, and experiments on the composition of the tryptophan-deficient basal diet, the consistency and repeatability of the assays, and other criteria of reliability have been performed. Results are given for the amounts of tryptophan utilized for weight gains and in certain

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<sup>2</sup> National Science Foundation Pre-Doctoral Fellow, 1955-1956. The material presented in this paper is taken from a thesis submitted to the Faculty of the Division of the Biological Sciences of The University of Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1956. Preliminary reports based on the results given were presented at the annual meetings of the American Institute of Nutrition, Fed. Proc., 15: 561 (1956); *ibid.*, 16: 591 (1957).

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untreated casein were analyzed for total nitrogen (macro-Kjeldahl), and the crude protein content ( $N \times 6.25$ ) calculated. The tryptophan content of the foods was assayed both microbiologically and chemically, and the results were in good agreement. Samples were hydrolyzed in 4 N NaOH for microbiological assay, using *Lactobacillus arabinosus* 17-5 (ATCC 8014) as the test organism. The Bates method ('37), as modified by Graham et al., ('47), was used for the colorimetric determination of tryptophan with *p*-dimethylaminobenzaldehyde. Thirty minutes at 40°C, in the dark, gave maximum color development.

One or more levels of each of the selected foods was added to the basal diet to provide known amounts of tryptophan (20 to 80 mg/100 gm) as calculated from the microbiological assay results. The percentage of tryptophan utilized for growth was calculated on the basis of the rates of gain for groups fed graded levels of L-tryptophan.

Fecal tryptophan excretion was determined in two feeding experiments. Composite collections of the feces of each group were made daily over a three-day period, and the tryptophan content assayed microbiologically, using *Lactobacillus arabinosus* 17-5 (ATCC 8014) as the test organism. Net food intake of each group was measured (gross intake minus spillage) and the total intake of tryptophan calculated for each group.

Experiments on the composition of the basal ration were conducted, in which variations in the amounts and proportions of oxidized and untreated casein were compared. Additional supplements of niacin and threonine alone and in combination were also tested.

#### RESULTS AND DISCUSSION

*Assay methods.* Results obtained with the use of the two tryptophan assay methods were within  $\pm 5\%$  of the mean values for all foods, and recovery experiments gave good results. The tryptophan-protein ratios were similar for all foods except split peas, and diets providing a chosen level of

excretion) gave no indication that tryptophan digestibility varied to any extent for the foods tested. These values ranged from 90% (split peas fed at a high level) to 99% digestibility (L-tryptophan fed in excess). When corrected for fecal tryptophan excretion on the basal ration (no tryptophan supplement added), tryptophan digestibility approximated 100% for all foods, ranging from 94 to 103%.

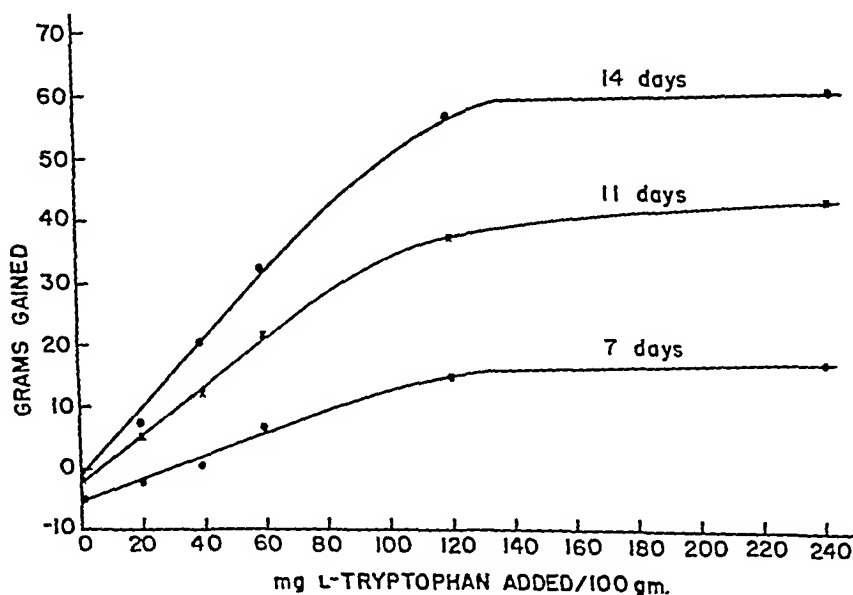


Fig. 1 Rates of gain for groups of male weanling rats fed the tryptophan-deficient basal ration plus graded tryptophan supplements for periods of 7, 11 and 14 days.

Experiments were conducted to ascertain whether modifications in the basal diet would provide increased rates of gain or result in altered tryptophan utilization values, and to give assurance that the basal ration was as complete as possible in all known nutrients. The results indicated that the nutrients other than protein (vitamins, minerals, fats, carbohydrate, and amino acid supplements) supported weight gains equivalent to those observed on the stock ration, when fed with 20 or 30% of untreated casein. It was concluded that the reduced

onine gave slightly *decreased* growth response, compared to that obtained with the unsupplemented basal ration when tested at the 40- and 80-mg levels of tryptophan supplementation. The results with added niacin and threonine in the basal ration showed decreasing tryptophan utilization with increasing levels of food supplements, an effect which was not observed in any of the feeding trials using the unsupplemented basal ration. This may have resulted from an amino acid imbalance caused by use of the threonine supplement.

TABLE 2

*Percentage of tryptophan utilized from several foods after 11- and 14-day experimental periods<sup>1</sup>*

FOOD TESTED	TRYPTOPHAN UTILIZED	
	11 days	14 days
	%	%
Raw ham (uncured)	113	103
Roast ham (uncured)	88	86
Unheated soybean meal	124	118
Normally processed soybean meal	125	125
Rolled oats	110	111

<sup>1</sup> Foods added to provide 40 mg of tryptophan/100 gm ration.

Thus, although the preliminary results described above indicated the potential value of adding additional niacin and threonine, these observations were not confirmed. It was concluded that the basal ration as originally designed was the one most satisfactory for determining the tryptophan utilization from foods.

*Utilization of tryptophan from foods.* The tryptophan utilization results obtained after 11 and 14 days are shown in table 2 for several of the foods studied, at the level of 40 mg of tryptophan/100 gm ration. These results indicate the high consistency observed in these studies for varying experimental periods. Results obtained after 7 days were more variable, and it appears desirable to study the quantitative utilization of tryptophan in the male weanling rat for a period somewhat longer than 7 days.

first experiment was obtained using 0, 20, 40, 60, 120, and 240 mg of L-tryptophan/100 gm ration. Thus the shape of the standard curve was not defined by experimental results in the region between 60- and 120-mg tryptophan supplement levels (see fig. 1). The weight gains observed for those foods fed at the 80 mg level of tryptophan supplementation were near the asymptotic portion of the growth curve and small differences in gain resulted in large differences in the calculated percentage utilization of tryptophan. All subsequent experiments included L-tryptophan at 80- and 100-mg levels as well, to provide additional information on the shape and configuration of the curve between the 60- and 120-mg levels.

TABLE 4

*Comparison of tryptophan utilization values observed in repeat experiments<sup>1</sup>*

FOOD TESTED	EXP. 1	EXP. 2	EXP. 6-a	EXP. 6-b <sup>2</sup>
Raw beef	68		112	104
Roast beef	82		108	89
Unheated soybean meal	152	118		
Normally processed soybean meal	138	125	116	123
Rolled oats	107	111	111	104

<sup>1</sup> Foods added to provide 40 mg of tryptophan/100 gm ration.

<sup>2</sup> Basal ration supplemented with 3 mg of niacin + 300 mg of DL-threonine/100 gm.

It was also important to determine the recovery of tryptophan when pure L-tryptophan was fed to provide 40 mg/100 gm ration in addition to an equivalent amount provided by each of several foods. Total tryptophan utilization values for L-tryptophan + test food of 95, 100, 111 and 103% were obtained for raw ham (uncured), unheated soybean meal, normally processed soybean meal, and rolled oats, respectively. These results indicate that tryptophan is readily utilized to support growth of the male weanling rat, whether obtained as the purified amino acid or as it occurs naturally in these food proteins.

Table 5 summarizes the values observed for the utilization of tryptophan from the foods tested in these studies, ranging

107% obtained for raw ham (uncured) is within the range of experimental error of the chemical and microbiological tryptophan assay methods and rat bioassay methods employed. The figures of 132, 121 and 117% observed for unheated soybean meal, normally processed soybean meal and rolled oats, respectively, suggest that the assay results for the tryptophan content of these foods may have been low. Other studies have shown that the presence of carbohydrates in foods may result in some destruction of tryptophan during hydrolysis of the sample, resulting in low values for tryptophan content. The use of alkali and cysteine is designed to minimize these losses in the hydrolysis procedures for microbiological analyses. Since the chemical and microbiological assay values agree, however, it is concluded that appreciable destruction of tryptophan did not occur during preparation of the samples for the microbiological assays. In the chemical method, the sample is merely solubilized by mild heating in alkali. The tryptophan values for the meats studied appear reliable, and the small amounts of carbohydrate present would not be expected to result in low values for the tryptophan assays. If the tryptophan assay results for the unheated and normally-processed soybean meals and rolled oats were too low by 10 to 15%, "true" tryptophan utilization values for these foods would lie close to 100%. It is also possible that tryptophan may be more effectively utilized from these food proteins than from L-tryptophan supplements.

The possibility that net synthesis or destruction of tryptophan by microorganisms in the intestinal tract might affect the results was considered. If this did occur, its effect was negligible in terms of the tryptophan utilization results obtained, in view of the consistency of the results for foods fed at several levels. Similarly, the possibility that such organisms might respond differently to tryptophan obtained from food proteins than to the pure amino acid was also considered, but the results of the recovery experiment indicate that any such differential response was of negligible effect on the tryptophan utilization values observed.

of niacin and threonine resulted in decreasing tryptophan utilization values as the amount of test food was increased.

The percentage of tryptophan utilized for growth of the male weanling rat when fed raw and roast beef, ham and lamb, nonfat dry milk, overheated soybean meal, and split peas ranged from 75 to 107%. Tryptophan utilization results for unheated and normally-processed soybean meals, and rolled oats ranged from 117 to 132%. The results suggest that cooking or heat processing may result in reductions in the availability of tryptophan to support growth. The percentage of ingested tryptophan excreted in the feces ranged from 1 to 10%.

The high values observed for tryptophan utilization in these studies gain added significance when compared to those observed in the earlier studies on lysine and methionine. The ranges in the observed amino acid utilization values for the foods tested were 49 to 98% for lysine, 48 to 83% for methionine, and 75 to 132% for tryptophan.

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POTENTIATING  
EFFECTS OF MATERIALS OF PLANT AND  
ANIMAL ORIGIN ON SYMPTOMS OF  
HYPERVITAMINOSIS A IN  
THE RAT<sup>1</sup>

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In recent years increasing interest has been shown in the study of hypervitaminosis A, the intoxication resulting from excessive intake of vitamin A. The subject is of interest not only because hypervitaminosis A has been observed in man but also because the condition serves as a useful tool for gaining insight into the metabolic behavior of vitamin A, particularly in regard to its interaction with other nutrients *in vivo* (Nieman and Obbink, '54). Available data indicate that the prolonged ingestion of massive doses of vitamin A results in growth retardation, spontaneous fractures, paralysis and other toxic manifestations in the immature rat, the severity of symptoms and their rapidity of onset being proportional to the dosage of vitamin A administered (See Rodahl, '50, and Nieman and Obbink, '54, for review of the literature). In the present communication data are presented indicating that alfalfa and other materials of plant and animal origin contain a factor or factors, apparently distinct from any of the known

<sup>1</sup>Communication 425 from the Department of Biochemistry and Nutrition, University of Southern California.

## RESULTS

*Experiment 1. Effects of graded levels of vitamin A acetate and vitamin A palmitate on symptoms of hypervitaminosis A in the rat*

In agreement with earlier findings (Rodahl, '50) the ingestion of massive doses of vitamin A resulted in a significant retardation in growth, the occurrence of spontaneous bone fractures in the legs and other manifestations of hypervitaminosis A toxicity including death. The severity and rapidity of onset of symptoms were proportional to the dosage of vitamin A administered. The ingestion of rations supplemented with 2.5 million U. S. P. units of vitamin A per kilogram of diet (either as the acetate or the palmitate) resulted in a slight retardation in growth but other manifestations of hypervitaminosis A toxicity including the occurrence of grossly detectable spontaneous leg fractures and paralysis were lacking. With the exception of a slight reduction in size these animals appeared grossly normal in all respects and were indistinguishable from rats fed the basal diet. The ingestion of diets supplemented with 3 or 3.75 million U.S.P. units of vitamin A per kilogram of diet (either as the acetate or the palmitate) resulted in a more pronounced retardation in growth and the occurrence of grossly detectable spontaneous leg fractures or paralysis or both in approximately 50% of the animals in these groups. The ingestion of diets supplemented with 5 million U.S.P. units of vitamin A per kilogram of diet (either as the acetate or the palmitate) resulted in a 100% incidence of leg fractures (and paralysis) and the death of 15 of the 16 rats in these groups within an experimental period of 6 weeks. No significant difference was observed at the various levels of supplementation between rats fed vitamin A acetate and those administered a comparable amount of vitamin A palmitate.

*Experiment 2. Potentiating effect of alfalfa meal on symptoms of hypervitaminosis A in the rat*

The findings indicate that supplements of alfalfa meal when fed at a 20% level in the diet resulted in a striking increase in

TABLE 1 (continued)

Comparative effects of alfalfa meal and other supplements on symptoms of hypervitaminosis A in the rat<sup>1,2</sup>

SUPPLEMENTS FED WITH BASAL DIET	NUMBER OF ANIMALS	INITIAL WEIGHT	GAIN IN BODY WEIGHT FOLLOWING DAYS OF FEEDING		PER CENT SURVIVING	RATS WITH FRAC- TURES <sup>3</sup>	AVERAGE TIME OF ONSET OF FRAC- TURE <sup>3</sup>
			21	42			
		gm	gm	gm		%	days
2.5 million units of vitamin A palmitate/kg of diet plus the following supplements:							
20% alfalfa meal, lot 3	8	42.0	40 (8)	51 (8)	100	100	17
20% defatted alfalfa meal <sup>4</sup>	8	42.3	46 (8)	58 (8)	100	100	21
B vitamins, C and K <sup>5</sup>	8	43.2	55 (6)	97 (6)	75	50	14
Vitamins A, D, and E <sup>6</sup>	8	43.4	54 (8)	96 (8)	100	37.5	24
10% casein <sup>7</sup>	8	42.2	60 (8)	105 (8)	100	50	24
10% cellulose <sup>8</sup>	8	43.0	55 (8)	106 (8)	100	37.5	21
5% corn oil	8	43.0	57 (7)	108 (7)	87.5	25	17
2.5% salt mixture <sup>10</sup>	8	42.3	63 (8)	111 (8)	100	25	17
Combined supplements <sup>11</sup>	8	42.0	60 (8)	103 (8)	100	50	21
2.5% alfalfa ash	8	42.5	64 (8)	115 (8)	100	12.5	29
Aureomycin HCl <sup>12</sup>	8	42.6	62 (8)	95 (8)	100	75	22
Mixed tocopherols <sup>13</sup>	8	42.4	59 (8)	114 (8)	100	25	23
EXPERIMENT 4							
None	8	42.3	72 (8)	121 (8)	100	0	
50% alfalfa meal, lot 4	8	42.0	77 (8)	119 (8)	100	0	
2.5 million units vitamin A palmitate/kg of diet	12	43.0	64 (12)	107 (12)	100	0	
3 million units vitamin A palmitate/kg of diet	8	43.0	54 (8)	94 (8)	100	12.5	22
2.5 million units of vitamin A palmitate/kg of diet plus the following supplements:							
5% alfalfa meal, lot 4	7	42.7	42 (7)	73 (7)	100	57	19
10% alfalfa meal, lot 4	7	43.0	43 (7)	66 (6)	86	86	16
20% alfalfa meal, lot 4	8	42.8	26 (8)	36 (3)	37.5	100	10
20% rye grass	7	42.5	32 (7)	60 (3)	43	86	15
20% orchard grass	7	42.9	28 (7)	66 (2)	29	86	17
20% wheat grass	7	43.0	32 (7)	49 (5)	71	100	21
20% fescue grass	7	42.6	44 (7)	60 (5)	71	86	19
20% oat grass	7	43.0	44 (7)	59 (7)	100	86	24
10% desiccated liver N.F.	8	42.2	45 (8)	80 (7)	87.5	100	14
10% liver residue	7	42.8	57 (7)	76 (7)	100	86	23
2.5% liver concentrate N.F.	7	43.0	52 (7)	90 (6)	86	71	14
10% yeast <sup>14</sup>	8	43.0	62 (8)	110 (8)	100	62.5	22
2.5% Vigofac <sup>15</sup>	7	43.0	45 (6)	76 (5)	71	71	13
5% tuna meal	8	42.6	64 (8)	105 (8)	100	0	
5% tuna solubles	8	42.8	52 (8)	96 (8)	100	25	24

<sup>1</sup> The values within parentheses indicate the number of animals which survived and on which averages are based.<sup>2</sup> The alfalfa samples were kindly provided by Dr. S. Tenkoff of Nutrilite Products, Inc., Buena Park, California. The hydrated rye grass, orchard grass, wheat grass, fescue grass and oat grass were obtained from the National Chlorophyll Chemical Company, Lamar, Colorado. The desiccated liver N.F., liver concentrate N.F. and liver residue were provided by Dr. David Klein of Wilson Laboratories, Chicago, Ill. Dr. E. Geiger of the Van Camp Sea Foods Company, Terminal Island, California, supplied the tuna meal and tuna solubles (50% solids). The vitamin A supplements employed in the present experiment were synthetic vitamin A acetate in corn oil (1 million U.S.P. units/gm) and synthetic vitamin A palmitate in corn oil (1 million U.S.P. units/gm), obtained from Hoffman-La Roche, Inc., Nutley, New Jersey.<sup>3</sup> Data were based only on animals with grossly detectable leg fractures sufficiently marked to result in dragging or disuse of the limb.<sup>4</sup> The water-washed alfalfa pulp remaining after the extraction of the juice.<sup>5</sup> Alfalfa meal was extracted for 15 hours with water followed by an 8 hour extraction with 50% acetone-water solvent. The acetone content of the resulting meal was 0.15% per gram.<sup>6</sup> The following vitamins were added per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 10 mg; nicotinic acid, 10 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; p-aminobenzoic acid, 40 mg; inositol, 50 mg; vitamin B<sub>12</sub>, 10 µg; and 2-methylerythritol, 5 mg. 1000 U.S.P. units of vitamin A, 500 U.S.P. units of vitamin D<sub>3</sub>, and 100 mg alpha-tocopherol acetate per kilogram of diet.<sup>7</sup> Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.<sup>8</sup> Solka-Flo HW 200, Brown and Co., Berlin, New Hampshire.<sup>9</sup> Hyal-B-N, New York; Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.<sup>10</sup> 10% calcium, 10% sodium, 10% corn oil, 2% salt mixture and 1% vitamin supplements in basal diet for studies 7 and 8.<sup>11</sup> 10% calcium, 10% sodium, 10% corn oil, 2% salt mixture.<sup>12</sup> The product employed was Aureomycin HCl, a gift of Dr. S. Tenkoff of Nutrilite Products, Inc., Buena Park, California.<sup>13</sup> The product employed was Vitamin E, a gift of Dr. S. Tenkoff of Nutrilite Products, Inc., Buena Park, California.<sup>14</sup> Vitaflo, Ciba Pharmaceutical Co., Easton, New Jersey.<sup>15</sup> Vigofac, Ciba Pharmaceutical Co., Easton, New Jersey.

had little if any potentiating effect. Alfalfa ash at a level corresponding to the amount provided by 20% alfalfa meal in the diet and mixed tocopherols at a level of 500 I.U. per kilogram of diet were also without significant effect. Aureomycin HCl at a level of 100 mg per kilogram of diet had little if any effect on weight increment but did appear to increase the incidence of leg fractures. The potentiation of the symptoms of hypervitaminosis A resulting from supplementation with alfalfa meal does not appear to be due to the ingestion of vitamin A precursors (carotenoids) in this material. This is indicated by the fact that the defatted carotenoid-free alfalfa meal was just as active as unextracted alfalfa meal in potentiating the symptoms of hypervitaminosis A in the present experiment. Furthermore, the severity of symptoms (i.e., growth retardation and incidence of leg fractures) obtained in animals fed the alfalfa supplements was significantly greater than that of animals fed the diet supplemented with 3 million U.S.P. units of vitamin A palmitate per kilogram of diet in spite of the fact that the latter ration contained 425,000 and 500,000 units more of vitamin A per kilogram of diet than the unextracted and defatted alfalfa meal rations, respectively.

*Experiment 4. Comparative effects of alfalfa meal and other materials of plant and animal origin on symptoms of hypervitaminosis A in the rat*

In agreement with previous finding supplements of alfalfa meal resulted in a striking increase in the toxic manifestations of hypervitaminosis A in the immature rat. The effects obtained were proportional to the alfalfa content of the ration. A supplement of 5% alfalfa meal significantly increased the growth retardation and incidence of leg fractures of rats fed the basal diet supplemented with 2.5 million U.S.P. units of vitamin A palmitate per kilogram of diet as compared to the results obtained on a similar ration with the alfalfa meal omitted. The effects obtained were less marked, however, than those which occurred at the 10% level of supplementation:

gross appearance. Rats fed the latter ration appeared normal in all respects and were indistinguishable grossly from the rats fed the basal diet.

#### DISCUSSION

The findings indicate that supplements of alfalfa and other succulent plants accentuated the symptoms of hypervitaminosis A in immature rats fed massive but relatively non-toxic doses of this vitamin. Desiccated liver, yeast, a product derived from fermentation sources and aureomycin HCl also showed some activity in this regard. In contrast to the above, supplements of all the known nutrients, either when fed alone or with one another, had little if any potentiating effect. These findings suggest that alfalfa and the other materials indicated above contain a factor (or factors) apparently distinct from any of the known nutrients which significantly increases the toxic manifestations of hypervitaminosis A in immature rats fed massive doses of this vitamin. No data are available to indicate the mechanism whereby the factor (or factors) indicated above exerts its physiologic effects. The possibility that alfalfa and the other active materials contain antioxidants that prevent the destruction of vitamin A in the diet and hence leave more of the vitamin present for absorption appears to be ruled out by chemical tests which indicate that no detectable destruction of vitamin A occurred in the purified basal diet supplemented with 2.5 million U. S. P. units of vitamin A palmitate per kilogram of ration after 30 days of refrigeration. Under conditions of the present experiment diets were made up at weekly intervals and were consumed within 10 days of the time they were made. In addition, no significant difference in response occurred between rats fed the above ration and animals fed an identical diet which was made up daily immediately before feeding. Furthermore, if an antioxidant effect were involved, one might have anticipated that the mixed tocopherols when fed at a level of 500 I. U. per kilogram of diet would have been active in potentiating symptoms of hypervitaminosis A. Such was not the case. The possibility that alfalfa and the other active materials contain

tion in experimental animals. A similar effect has been reported for aureomycin (Murray and Campbell, '55a, b). That at least two separate factors exist in the above supplements would seem to be indicated by the observation that the yeast factor is soluble in fat solvents (Patrick and Morgan, '43) whereas the fish factor is water-soluble (Harms et al., '56b). Whether alfalfa and the other materials which potentiated symptoms of hypervitaminosis A in the present experiment would increase the absorption or utilization of vitamin A when the latter is ingested in suboptimal amounts has not been determined. It is of interest, however, that both yeast and aureomycin which were effective in increasing the absorption or utilization of suboptimal amounts of vitamin A (Patrick and Morgan, '43; Murray and Campbell, '55a, b) resulted in a significant increase in the incidence of fractures in rats fed a massive but relatively non-toxic dose of vitamin A under conditions of the present experiment.

#### SUMMARY

Immature rats were fed a purified ration containing a massive but relatively non-toxic dose of vitamin A. Supplements of alfalfa meal and other succulent plants resulted in a significant potentiation of the symptoms of hypervitaminosis A. Both the dried alfalfa juice and the water-washed pulp remaining after the extraction of the juice were active in this regard. Desiccated liver, yeast, a product derived from fermentation sources<sup>8</sup> and aureomycin HCl also showed activity. In contrast to the above, supplements of all the known nutrients had little if any potentiating effect.

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<sup>8</sup> See footnote 5, p. 534.

## NUTRIENTS AFFECTING THE VITAMIN B<sub>12</sub> REQUIREMENT OF CHICKS<sup>1</sup>

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It has been recognized from the time of the early studies with vitamin B<sub>12</sub> that the requirement for this vitamin may be influenced by other nutrients in the diet. Schaefer and co-workers ('49) reported that choline could lower the vitamin B<sub>12</sub> requirement of chicks from hens receiving a standard breeder diet. Thus, these chicks did not have low vitamin B<sub>12</sub> stores. Briggs et al. ('50) reported that the growth of nondepleted chicks was stimulated by supplemental choline or methionine in the absence of vitamin B<sub>12</sub>. Sunde et al. ('51) observed vitamin B<sub>12</sub>-sparing effects with choline and methionine in chicks fed a peanut oil meal basal ration. Machlin et al. ('52) observed similar sparing effects with methionine in chicks fed a vitamin B<sub>12</sub>-free corn-soybean oil meal diet. Some workers, however, have failed to obtain a sparing effect upon the vitamin B<sub>12</sub> requirement with methionine (Jukes and Stokstad, '51; Titus et al., '55); it appears that a sparing effect might have occurred if higher supplemental levels of methionine had been used. Early studies of the "animal protein factor," which are pertinent to the general topic of vitamin B<sub>12</sub> activity as affected by constitution of the diet, have been reviewed by Briggs et al. ('50).

<sup>1</sup>A preliminary report of some of these data was given at the annual meeting of the American Institute of Nutrition, Atlantic City, April 12-16, 1954.

components. The C2 concentrate <sup>4</sup> (diet C2 less carbohydrate and vitamin B<sub>12</sub>) was substituted for corn in diet C30; various incomplete C2 concentrates were also fed, as indicated in table 1. The amino acid mixture AA5,<sup>5</sup> which was used in some experiments, was formulated to simulate the amino acid composition of casein.

In some experiments the chicks were decapitated and the livers, pooled by experimental group, were assayed for vitamin B<sub>12</sub> with *Lactobacillus leichmannii* by the U. S. P. procedure ('55).

### RESULTS

The data in table 1 are from one experiment that demonstrates the typical effects observed upon supplementing the basal corn-soybean oil meal diet with vitamin B<sub>12</sub> and various ingredients of the C2 diet. Chicks receiving the basal diet alone grew very poorly during the 4-week period, utilized the diet inefficiently, and had low stores of vitamin B<sub>12</sub> in the liver. The addition of 100 µg of vitamin B<sub>12</sub> per kilogram of diet improved growth, feed efficiency, and elevated the concentration of vitamin B<sub>12</sub> in the liver. Inclusion of 219 gm of C2 concentrate (which contained no vitamin B<sub>12</sub>) per kilogram of basal diet C30 markedly improved growth and feed efficiency but did not raise the concentration of vitamin B<sub>12</sub> in the liver. The C2 concentrate, from which methionine was omitted,

<sup>4</sup> When the C2 concentrate was incorporated into diet C30, it furnished the following per kilogram of diet: casein 100 gm, gelatin 40 gm, DL-methionine 1.5 gm, corn oil 20 gm, chick salts A 30 gm, glucose 18.5 gm (carrier for the B vitamins), thiamine hydrochloride 4 mg, riboflavin 4 mg, calcium pantothenate 10 mg, choline chloride 1000 mg, nicotinic acid 50 mg, pyridoxine hydrochloride 4 mg, d-biotin 0.15 mg, pteroylglutamic acid 1.5 mg, vitamin A acetate 1.5 mg, vitamin D<sub>3</sub> 0.01 mg, α-tocopherol acetate 5 mg, and 2-methyl-1,4-naphthoquinone 0.5 mg.

<sup>5</sup> When amino acid mix AA5 was incorporated in the diet at a level of 5%, it contributed the following quantities of amino acids in grams per kilogram of diet: DL-alanine 1.56, L-arginine hydrochloride 1.61, DL-aspartic acid 2.57, L-cystine 0.22, L-glutamic acid 2.65, glycine 0.88, L-histidine hydrochloride 1.26, DL-isoleucine 5.15, DL-leucine 4.16, L-lysine hydrochloride 2.45, DL-methionine 1.53, DL-phenylalanine 2.96, DL-proline 2.31, DL-threonine 4.03, L-tryptophan 0.52, L-tyrosine 2.03, and 100 units of B<sub>12</sub>.



caused an increase in growth; however, the 4-week weight of this group was poorer than that of chicks receiving the complete C2 concentrate. When both methionine and the B vitamins (including choline) were omitted from the C2 concentrate, growth was not increased significantly over that of the basal group. The omission of the fat-soluble vitamins and gelatin from the supplemental C2 concentrate did not significantly alter the good growth supported by the complete concentrate. The data thus far indicate that methionine and one or more of the B vitamins were the chief constituents in the diet C2 that spared vitamin B<sub>12</sub>.

The simultaneous incorporation of vitamin B<sub>12</sub> and the complete C2 concentrate into the basal diet resulted in a very marked improvement of growth. It has been reported previously (Fox et al., '56) that 100 µg of vitamin B<sub>12</sub> per kilogram of diet supported a maximal growth response with this diet; therefore, the improved growth with both supplements over that with only vitamin B<sub>12</sub> indicated that this basal corn-soybean oil meal diet was somewhat deficient in nutrients other than vitamin B<sub>12</sub> for optimal growth of the young chick.

In table 2 data are presented from 5 series of experiments on the vitamin B<sub>12</sub>-sparing capacities of individual nutrients and combinations of nutrients present in the C2 concentrate. The column headed "Average" is the mean weight and standard error of the total number of chicks from all series that were fed the indicated supplement. Since all supplements were not included in each series, evaluation of the average values must be tempered by the data of the specific series involved. Mortality in all groups was negligible. When the fat-soluble vitamins, the complete B vitamin mix, or the B vitamin mix minus choline was incorporated in the basal diet, there was no improvement in growth. The addition of 0.2% choline chloride always resulted in mean weight gains. These weight increases were not statistically significant on the basis of comparisons within each series; however, upon consideration

of all 5 series together choline did cause a significant increase in weight gain.

The addition of 0.15% methionine to the basal diet caused marked improvement of growth, which was as good as that with vitamin B<sub>12</sub>. Data obtained from feeding levels of methionine below 0.15% down to 0.05% were not presented in table 2 since the increases in growth were quite variable. These results may be related to slight variations in the methionine content of crude materials in the diet. Supplementary cystine was completely without effect upon growth of vitamin B<sub>12</sub>-deficient chicks. The effect of feeding the combination of choline and methionine was little different from the result due to methionine alone. Feeding 5% of casein, which should contribute approximately 1.5 gm of methionine per kilogram of diet, did not improve the growth of vitamin B<sub>12</sub>-deficient chicks to the same extent as this level of free methionine. In two of the 5 series the casein caused significantly improved growth; however, the combination of casein and choline supported growth that in general was superior to that attained with either alone.

The amino acid mix AA5, whose composition was based on the amino acid content of casein, stimulated growth of vitamin B<sub>12</sub>-deficient chicks in a manner similar to that of the same level of casein. Again, the combination with choline was superior to either alone. Omission of methionine from the amino acid mix caused a marked depression of growth of the vitamin B<sub>12</sub>-deficient chicks, but feeding vitamin B<sub>12</sub> with the methionine-free amino acid mix resulted in growth similar to that of the control group receiving vitamin B<sub>12</sub> alone. The addition of 0.2% choline chloride at least partially overcame the growth depression caused by the methionine-free amino acid mix.

Many of the supplements that supported improved growth in the deficient chicks were also fed in the presence of vitamin B<sub>12</sub>. The growth increments caused by the various supplements in the presence of vitamin B<sub>12</sub> were much smaller than the growth increments of the supplements seen in the absence

rather than to limited availability of the methionine. The amino acid mix AA5, which was based on the composition of casein, elicited growth responses under various conditions comparable to those with the intact protein. In addition, the same amino acid mix minus methionine depressed growth below that obtained with the basal diet alone. This nutritional stress is comparable to that of high fat in that either methionine or vitamin B<sub>12</sub> could overcome the effect. The growth depression observed with the methionine-free amino acid mix may be due to an amino acid imbalance involving specific amino acids, or it may be a non-specific increase in methionine requirement associated with increased protein in the diet.

The combination of choline with casein or the complete amino acid mix was similar to the equivalent amount of free methionine fed alone. Under these conditions, choline may have been counteracting the suppression of methionine by the other amino acids present, or it may have been exerting some other beneficial effect unrelated to the amino acid content of the diet.

Diet C30 is limiting chiefly in methionine; therefore, when determining the vitamin B<sub>12</sub> potency for the chick of crude concentrates or compounds related to vitamin B<sub>12</sub>, it is necessary to keep the methionine content of the material being assayed at a minimum. On the other hand, it is desirable to limit the methionine content of the diet to the lowest possible level consistent with good growth in order to obtain the greatest growth stimulation upon addition of vitamin B<sub>12</sub>.

#### SUMMARY

The effect of certain constituents of purified diets upon the vitamin B<sub>12</sub> requirement of chicks fed a high fat corn-soybean oil meal diet has been studied. It is concluded that:

1. The only nutrients having a significant vitamin B<sub>12</sub>-sparing activity were methionine and choline. Methionine at a level of 0.15% completely replaced vitamin B<sub>12</sub>, whereas addition of 0.2% choline chloride to the diet resulted in only a small sparing effect.

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## UTILIZATION OF FOOD FOR WEIGHT MAINTENANCE AND GROWTH

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The problems of how food is utilized under various conditions have been discussed by nutritionists for many years. Practically every worker in this field has made contributions to these questions. A new facet of the old problem was revealed in studies of the food requirements for weight maintenance and growth of rats kept at constant weight by restricted feeding. As previously shown (Kaunitz et al., '56a, b), measurements of food and water intake and organ weights of such animals disclosed the existence of certain adaptive mechanisms. It therefore seemed worth while to study food utilization for weight maintenance and growth of rats of various ages and weights after different periods of food restriction. The findings are discussed in this paper.

### PROCEDURE

The male albino rats used for the experiments were drawn from a homogeneous colony known to us for 15 years. When they were 20 days old and weighed 45 to 50 gm, they were placed on a complete stock diet containing 30% protein. They were weighed at 24 and at 28 days, and, at the latter time, those of suitable weights (usually about two-thirds of the total) were divided into matching groups of 8 animals whose average weights at 24 and again at 28 days were identical. When these groups were permitted to eat freely, their average

TABLE 1  
Average food requirements of male rats for maintenance of body weight and growth at different ages and body weights and after different periods of restricted food intake

FIRST 3 DAYS OF RESTRAINT									
GROUP	AGE	PREVIOUS RESTRAINT	BODY WT.	Maintenance requirement		Total food consumed	Increase in body wt.	Require- ment per gm increase	Maintenance requirements for successive weeks of restriction
				Per gm body wt. per wk.	Total for 3 days (calculated)*				
wt.	wt.	wt.	gm	gm	gm	gm	gm	gm	gm
Series I									
1	9	4	97 ± 3*	0.37	17.2 ± 1.2	38.1 ± 1.9	23 ± 4	0.91	0.51, 0.42, 0.38, 0.37
2	9	3	122 ± 9	0.38	21.9 ± 1.4	44.7 ± 6.0	26 ± 5	0.84	0.49, 0.36, 0.37
3	9	2	151 ± 17	0.39	27.1 ± 3.2	44.7 ± 7.0	22 ± 5	0.80	0.44, 0.39
4	9	1	177 ± 13	0.43	34.2 ± 2.7	48.7 ± 4.4	19 ± 4	0.76	0.43
5	9	0	214 ± 12	0.37	33.2*	43.2 ± 2.9	10 ± 4	1.00	...
Series II									
1	5	1	61 ± 2	0.51	15.1 ± 0.8	29.8 ± 1.7	16 ± 3	0.93	0.51
1	5	1	95 ± 6	0.50	21.6 ± 1.4	32.6 ± 2.6	11 ± 2	1.00	0.50
3	6	1	109 ± 6	0.46	22.2 ± 1.1	29.5 ± 2.4	7 ± 3	1.04	0.46
2	13	1	180 ± 15	0.40	31.9 ± 2.8	43.7 ± 5.1	12 ± 3	0.98	0.40
1	13	2	167 ± 13	0.33	24.5 ± 1.8	36.5 ± 4.2	11 ± 3	1.09	0.40, 0.33
6	13	1	151 ± 15	0.32	22.3 ± 2.1	40.9 ± 6.8	16 ± 4	1.16	0.44, 0.37, 0.30, 0.32,
5	13	6	128 ± 14	0.33	19.7 ± 2.0	39.6 ± 6.5	19 ± 3	1.05	0.41, 0.40, 0.36, 0.33, 0.31, 0.33

\* Eight animals per group.

\* Obtained by multiplying the average body weight for the three-day period by 3/7 of the food requirement per gram of body weight as observed in the preceding week.

\* Average ± standard deviation.

\* Calculated by second method described in the text.

weight was 97 gm and from 0.41 to 0.33 gm for the group in the second series which was kept at 128 gm for 6 weeks.

When the animals were restricted for one week at different ages and weights, the requirements for the maintenance of one gram of body weight declined with increasing body weight. This is evident from the data from the second series, where the average body weight increased from 61 to 180 gm (column 4) and the maintenance requirements per gram of body weight decreased from 0.51 to 0.40 (column 5). This was also true for the animals in the first series when the requirements were calculated after one week of food restriction at different weight levels. The average body weight varied, among 4 groups, from 95 to 177 gm, and the maintenance requirements decreased from 0.51 to 0.43 gm.

The decreasing maintenance requirements for higher body weights are in agreement with many studies of the relation of food requirements to body surface in that heavier animals have a relatively smaller surface. It is not probable that the differences in age in these experiments were of consequence because animals restricted for a short period earlier in the experiments and having, thus, comparatively reduced body weights had requirements similar to those of younger animals of comparable weights which had eaten freely throughout the experiment.

*Requirements for weight increase.* For the determination of the requirements for weight increase, the groups which had been restricted for varying periods of time and then permitted to eat freely were used. The weight gain and food consumption of only the first three days of realimentation were used because, on the one hand, the period was long enough for accurate measurements and, on the other hand, was short enough to permit the assumption that the basic requirement for the maintenance of one gram of body weight would not have changed with the return to "luxury" food consumption. The rats' maintenance requirements for this three-day period were calculated by multiplying the requirement at the lower body weight, as known from the previous week's restriction,

groups. The total requirements for weight maintenance during the first week of food restriction of two well-matched groups whose restriction had begun on successive weeks were averaged because the similarity in the requirements of different groups made it permissible to assume that the value thus obtained would be approximately that of any well-matched freely eating group of the same series over the same weight range. This was now deducted from the total food intake of the freely eating group, and the difference was divided by the gain in body weight to give the amount required for one gram of weight increase. The values obtained by this procedure varied, with one exception, from 0.75 to 1.25 gm of food for one gram of body weight increase and were in fair agreement with the data obtained by the first method. In the table, only the values obtained by the first method are given with the exception of the value for group 5, series I, which was calculated by the second method.

When considering errors in the determination of the requirements for weight maintenance and for weight increase, it must be kept in mind that the calculation of the latter is based on three variables, namely, weight maintenance requirements, weight gain, and total food consumption. This contributes to greater variability in the values for weight increase requirements than in those for weight maintenance requirements because the latter are merely based on the determination of the daily food requirements. For this reason and also because we have noted considerable constancy in 15 experimental series with several groups in each one, differences of as little as 5 to 10% are usually significant in the determination of weight maintenance requirements. In the determination of the requirement for weight increase, the data indicated that, in all probability, only differences exceeding 25% are significant. Direct statistical analyses of the weight increase requirements are difficult in view of the above-noted three variables.



of rats kept at constant weight by restricted feeding of a complete, purified diet and of the food intake and weight increase of well-matched rats permitted to eat freely of the same diet.

2. The requirements for the maintenance of one gram of body weight were found to be influenced by the duration of food restriction, the body weight, and the room temperature but hardly by the age of the animals. Continued food restriction led to a reduction of about 30% in the weight maintenance requirements.

3. Food requirements for weight increase did not seem to be influenced by the above factors. Approximately one gram of food was necessary to build one gram of body substance under widely different conditions.

4. The implications of these findings for studies of food utilization and paired weighing and paired feeding techniques are discussed.

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# THE NON-ESSENTIALITY OF FLUORINE IN NUTRITION<sup>1,2,3</sup>

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Fluorine occurs naturally in virtually all foods and drinking water and it is present in the bodies of all higher animals. Even though the element is of practical significance in the partial protection of teeth from dental caries, conclusive studies have not been reported concerning its essentiality in nutrition.

Sharpless and McCollum ('33) investigated the essentiality of fluorine for rats using a semipurified diet containing casein, starch, butterfat, yeast, and salts. The femurs of the animals contained about 150  $\mu$ g of fluorine per gram at 120 days of age, indicating that appreciable amounts of the element remained in the diet. Under these conditions there was no indication that fluorine is essential.

Phillips, Hart and Bohstedt ('34) fed rats a mineralized milk diet reported to contain only 0.1 to 0.2 ppm of fluorine. Nevertheless, at 140 days of age the animals contained several hundred micrograms of the element. Evans and Phillips ('39)

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<sup>2</sup> The material for this paper was taken from a thesis submitted to the faculty of the Graduate School in partial fulfillment of the requirements for the degree, Doctor of Philosophy, in the Department of Chemistry, Indiana University, by R. L. Maurer.

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samples of material when the concentration of fluorine was very small.

In the analysis of samples containing large amounts of salts a single Willard and Winter ('33) distillation did not suffice to separate all the fluorine from the elements which interfere in the determination. Savchuck and Armstrong ('51) had already noted the necessity of employing a double distillation procedure for the determination of fluorine in some cases. The technique employed here was to collect 200 ml of distillate during the primary distillation and then return this to the rinsed still for evaporation to near dryness with highly purified calcium oxide (Weddle and Maurer, '54). A second distillation was then run. Samples were ashed in silica or platinum dishes, first over a Meker burner and then for 8 hours at 550 to 600°C. in a muffle furnace fitted with a silica lining to prevent contamination.

Alkaline and acid phosphatase were determined according to the procedure of King and Armstrong ('34).

*Experimental diet.* The diet was composed of casein, 18; DL-methionine, 0.5; corn oil, 15; vitamin mixture, 1; salts, 4; and corn starch, 61.5%. The composition of the salt mixture was, in grams, sodium chloride, 293; monopotassium phosphate, 817; calcium oxide, 488; magnesium sulfate, 120; ferric sulfate, 138; manganese sulfate, 9; zinc sulfate, 3; cupric sulfate, 1; potassium iodide, 2; molybdc oxide, 2. The vitamin mixture contained thiamine hydrochloride, 1400 mg; inositol, 1400 mg; calcium pantothenate, 700 mg; pyridoxine hydrochloride, 700 mg; riboflavin, 1400 mg; nicotinic acid, 1400 mg; folic acid, 350 mg; menadione, 350 mg; vitamin B<sub>12</sub>, 14 mg; biotin, 2 mg; choline chloride, 42 gm; corn starch carrier, 302 gm. The vitamins were all the purest crystalline materials available commercially and were used without further purification. Each 15 gm of corn oil was fortified with 2500 units of vitamin A and 360 units of vitamin D added as perccormorph oil, and 15 mg of  $\alpha$ -tocopherol.

All equipment used in the purification, storage, compounding and dispensing of the diet was cleaned by boiling in either

quently for the two hours required to evaporate all traces of acetone.

Sodium chloride, monopotassium phosphate, and cupric sulfate were purified by multiple recrystallizations from redistilled water. Calcium oxide was prepared according to the procedure of Weddle and Maurer ('54), and contained no more than 0.2 ppm of fluorine. Magnesium, ferric, zinc, and manganous salts were purified by procedures involving recrystallizations from redistilled water and ignition to red heat with sulfuric acid. Molybdic acid was dissolved with the aid of  $\text{NH}_4\text{OH}$ , filtered, reprecipitated with  $\text{HCl}$ , and ignited to red heat. Potassium iodide was purified by fractional recrystallizations from redistilled absolute ethanol.

*General care of animals.* The animals were housed individually in special round metabolism cages mounted on pyrex cake pans. The cages were constructed entirely of stainless steel, and were fabricated by techniques which eliminated the use of welding and soldering fluxes containing fluorine. The bottoms were 8 inches in diameter and made of 3-mesh stainless steel wire. The excreta required for analysis were collected on filter paper circles,<sup>5</sup> which had an especially low fluorine content. The caged animals were kept in a special air-tight chamber supplied with air washed with water. This was necessary owing to the presence of some fluorine at all times in unfiltered or unwashed air. The temperature and humidity were controlled at approximately 25°C. and 70%, respectively. All fluorine-containing materials except fluoridized water for the control animals were rigorously excluded from the room.

The experimental animals were obtained in the following manner: a pair of weanling albino rats of the Wistar strain was obtained from a dealer and placed on a stock corn diet (Muhler and Day, '51) and redistilled water. The diet contained only about 0.6 ppm of fluorine. When sexually mature, the animals were mated and their pups in turn were raised to maturity on the corn diet and then transferred to clean cages,

<sup>5</sup>CS and S filter paper no. 470.

TABLE 1

*Growth of fluorine-deficient rats compared with controls given sodium fluoride<sup>1</sup>*

GENERATION AND SEX	FLUORINE ADDED	AGE IN DAYS											
		25	35	45	55	65	75	85	95	105	120	150	
1	M	—	66	119	176	237	294	319	312	348	358	374	434
1	M	—	..	101	151	213	261	288	284	313	324	353	380
1	M	+	56	96	160	215	268	317	307	347	355	375	395
1	M	+	..	128	184	215	270	304	311	322	325	338	363
1	F	—	62	107	142	159	178	199	205	209	213	224	..
1	F	—	58	100	138	158	178	198	193	..	..	221	..
1	F	—	36	103	..	171	185	197	212	214	..	..	..
1	F	+	54	100	134	153	178	192	231	241	242	246	250
2	M	—	51	110	..	222	253	286	298	320	330	325	357
2	M	—	63	128	172	244	280	311	327	345	352	374	395
2	M	+	59	116	166	229	270	295	308	324	340	385	405
2	F	—	52	104	138	158	177	185	185	196	..	..	..
2	F	—	45	94	119	136	155	178	166	170	197	..	..
2	F	—	42	94	130	164	186	212	226	234	..	..	259
2	F	—	37	84	119	143	160	178	188	200	..	..	220
2	F	—	56	97	129	145	..	185	187	195	211	227	221
2	F	—	46	91	135	145	164	172	180	178	193	..	220
2	F	+	55	98	126	150	173	189	194	204	214	..	..
2	F	+	54	99	136	..	183	194	195	199	203	..	..
2	F	+	45	94	134	160	178	196	196	200	214	..	258
3	M	—	49	108	143	202	250	285	300	295	273	..	..
3	M	—	50	115	161	227	276	309	307	313	310	..	..
3	M	—	42	110	158	238	283	310	320	332	355	..	..
3	M	+	53	117	173	220	275	287	300	314	289	..	..
3	F	—	46	93	117	138	150	173	170	170	180	..	..
3	F	—	46	100	123	154	161	189	180	189	170	..	..
3	F	+	50	102	132	160	171	190	200	206	205	..	..
Mean Males (7)		—	54	112	160	225	271	301	307	324	329	356	392
Males (4)		+	56	114	170	220	271	301	306	326	327	366	388
Fem. (11)		—	48	97	129	152	169	187	194	195	194	..	..
Fem. (5)		+	52	99	132	156	176	192	203	210	215	..	..

<sup>1</sup> The weights of the animals are given in grams.

ages. No distinction was made according to generation, since the pups in the first generation, as well as in subsequent generations, were free of detectable amounts of fluorine until they were over 10 days of age. Such fluorine as was present in the animals must therefore have been accumulated from

TABLE 2

*Phosphatase activity of tissues of deficient and supplemented animals*

AGE	SEX	FLUORINE	ALKALINE PHOSPHATASE <sup>1</sup>			ACID PHOSPHATASE <sup>1</sup>		
			Kidney	Liver	Bone	Kidney	Liver	Bone
<i>days</i>								
105	M	—	183	.	86	15	18	10
105	M	+	155	1.2	63	14	16	13
125	F	—	198	0.4	61	20	14	9
125	F	+	149	0.8	38	15	13	18
130	F	—	198	0.9	61	14	17	12
130	F	+	190	2.6	82	17	20	16
145	F	—	207	0.6	66	15	12	10
145	F	—	299	0.7	29	17	12	4
145	M	—	94	0.1	78	20	14	..
145	M	+	122	0.6	81	22	16	..
153	F	—	291	1.1		25	18	..
153	F	+	184	1.2		24	16	..
160	F	—	235	1.0	66	27	13	8
160	F	+	140	1.0	62	22	9	7
190	F	—	252	1.2	55	22	15	9
210	M	—	183	1.2	51	25	11	6
325	M	—	120	0.6	38	24	12	9
325	M	+	150	1.1	35	21	12	8
325	F	—	226	0.5	46	26	15	6
325	F	+	167	0.5	51	17	12	5
Av.		—	210	0.70	62	21	15	9
S.d.			59	0.28	15	4.8	2.2	1.7
Av.		+	174	0.92	55	19	14	16
S.d.			28	0.28	19	4.0	3.3	5.3

<sup>1</sup> Milligrams of phenol liberated from disodium phenylphosphate per hour per gram of tissue.

the diet, the drinking water, and the environment. A few fluorine determinations were made on the femora of older animals for purposes of comparison. It should be noted that even at 160 days of age the fluorine content of both femora was often too small to be measured. Even the rats that had been on the diet 325 days had only approximately 2 ppm fluorine in the femora. The whole carcasses, including skin and hair, or femora of over 33 rats on the fluorine-deficient regimen were analyzed. The results indicated considerable variability in the amounts of fluorine present but in only two or three cases were the amounts high enough to suggest that less than maximum care had been exercised to prevent appreciable contact with the element.

#### DISCUSSION

Under the extremely rigorous conditions of this study fluorine was not found to have any influence on the growth and well-being of rats. There were not even any grossly detectable dental defects. Thus it is justifiable to conclude that under some conditions fluorine may not have any value in nutrition or even in the maintenance of dental health. Concerning dental caries, it should be noted that the experimental diet was sugar free and it was not conducive to impaction of food in the fissures of the teeth. Thus, on the basis of the current views regarding dental caries (Muhler, Hine and Day, '54), fluorine would not be expected to play an important role in the protection of the teeth from decay under the conditions of this experiment.

Several attempts were made to analyze the compounded diet for fluorine, but the content was apparently so low that the analytical method would not measure it accurately. Therefore, the amount of fluorine which accumulated in the experimental rats became an important criterion of the success in producing a diet and environment extremely low in fluorine.

When the total amount of fluorine is so extraordinarily small, probably of greater importance is the amount that is utilizable. If it is assumed that a 150-day-old animal con-

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## STUDIES ON THE PROTEIN QUALITY OF HIGH-OIL, HIGH-PROTEIN CORN

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Several recent studies have been made on the nutritive value of high-protein corn. Flynn, Zuber, Leweke, Grainger and Hogan ('54) and Sauberlich, Chang and Salmon ('53) reported that the percentages of lysine and tryptophan in the protein decreased with the increase in the protein content of corn. The decrease in the relative amounts of these essential amino acids is usually explained on the basis that in high-protein corn the poorer quality zein protein contained in the horny endosperm increases at a faster rate than the high quality protein of the germ portion of the corn.

Mitchell, Hamilton and Beadles ('52) found that for rats the biological value of the protein decreased considerably as the protein content of the corn increased. Eggert, Brinegar and Anderson ('53) concluded that high-protein corn was superior to low-protein corn when fed to pigs; however, the protein quality of low-protein corn was found to be better than that of high-protein corn. Ross, Carigus, Hamilton and Earley ('54) found that the high- and medium-protein corns gave better weight gain and wool production in lambs. Schulz and Thomas ('49) fed the germ and endosperm from waxy and starchy inbred corns of different genetic constitution to rats for determination of a biological value based on nitrogen balance experiments. No highly significant differences were noted in biological values among these germ and endosperm samples.

TABLE 1  
*Analysis of corn samples<sup>1</sup>*

SAMPLE	PROTEIN <sup>2</sup>	FAT <sup>2</sup>	MOISTURE
	%	%	%
High-oil corn	13.0	8.6	10.2
Three-way cross corn	11.5	7.0	11.5
Regular yellow corn	9.3	5.1	14.0
High-oil corn grits	11.8	2.3	11.6
Regular corn grits	8.2	1.2	9.7
High-oil corn germ	13.1	27.5	6.7
Regular corn germ	14.6	20.7	2.3

<sup>1</sup> Values for grits and germ obtained from commercial dry-process separation.

<sup>2</sup> Calculated on dry basis.

TABLE 2  
*Composition of test diets*

DIET	RATION COMPONENTS						
	Corn	Mineral <sup>1</sup> mix	Corn-starch	Corn oil	Dex-trose	Alpha-cel	Vitamin <sup>2</sup> mix
	%	%	%	%	%	%	%
Group I							
High-oil corn	95.8	4.0					0.2
Three-way cross	95.8	4.0					0.2
Regular corn	95.8	4.0					0.2
Group II							
High-oil corn	64.5	4.0	30.3	1.0			0.2
Three-way cross	73.9	4.0	20.5	1.4			0.2
Regular corn	94.2	4.0		1.6			0.2
Group III							
Regular corn germ	81.7	4.0	6.0	8.1			0.2
High-oil corn germ	95.8	4.0					0.2
Regular corn grits	90.9	4.0		4.9			0.2
High-oil corn grits	64.7	4.0	27.1	4.0			0.2
Group IV							
Nitrogen-free		4.0		6.0	87.8	2.0	0.2
High-oil	95.8	4.0					0.2
Regular corn	93.9	4.0		1.9			0.2

<sup>1</sup> U.S.P. Salt Mix XIV.

<sup>2</sup> Vitamins added per 1,000 gm of each of the diets: 2 methyl-naphthoquinone, 2 mg; thiamine hydrochloride, 12 mg; pyridoxine hydrochloride, 20 mg; biotin, 0.3 mg; vitamin B<sub>12</sub>, 1 mg; folic acid, 0.9 mg; calcium pantothenate, 50 mg; nicotinic acid, 90 mg; inositol, 200 mg; p-aminobenzoic acid, 300 mg; riboflavin, 50 mg; choline chloride, 1,000 mg; dl-ascorbyl-2-polyphosphate, 100 mg. These vitamins were diluted to 2 gm with cornstarch before addition to the diets.

TABLE 3  
Summary of test data

DIET	PROTEIN CONTENT OF DIET	AVERAGE FOOD EATEN	AVERAGE PROTEIN EATEN	AVERAGE BODY WEIGHT GAIN	AVERAGE PROTEIN EFFICIENCY <sup>1</sup>
	%	gm	gm	gm	
Group I					
High-oil corn	10.8	264.9	28.6	38.6 ± 4.2 <sup>2</sup>	1.33 ± 0.09 <sup>2</sup>
Three-way cross corn	9.8	275.7	27.3	37.8 ± 3.1	1.39 ± 0.07
Regular corn	8.4	254.8	21.4	19.1 ± 4.3	0.89 ± 0.18
Group II					
High-oil corn	7.3	181.8	13.3	6.7 ± 1.7	0.51 ± 0.13
Three-way cross corn	7.5	183.1	13.7	9.4 ± 2.2	0.69 ± 0.14
Regular corn	7.7	181.9	14.0	5.5 ± 1.7	0.40 ± 0.12
Group III					
Regular corn germ	11.8	254.5	30.0	64.5 ± 5.2	2.15 ± 0.06
High-oil corn germ	12.1	248.3	30.0	52.1 ± 1.9	1.74 ± 0.05
Regular corn grits	6.9	185.8	12.8	2.3 ± 1.0	0.18 ± 0.09
High-oil corn grits	7.1	180.7	12.8	0 ± 0.6	..

<sup>1</sup> Protein efficiency calculated as grams gain in weight per gram of protein eaten.

<sup>2</sup> Standard error of mean.

TABLE 4  
Significance of means

DIETS COMPARED	BODY WEIGHT GAIN		PROTEIN EFFICIENCY	
	"t" value	P	"t" value	P
Group I				
High-oil vs. regular corn	3.24	< 0.01	2.31	< 0.05
Three-way cross vs. regular corn	2.99	< 0.02	2.39	< 0.05
Group II				
High-oil vs. regular corn	0.50	> 0.1	0.62	> 0.1
Three-way cross vs. regular corn	1.33	> 0.1	1.37	> 0.1
Group III				
Regular corn germ vs. high-oil corn germ	2.24	< 0.05	4.02	< 0.01
Regular corn grits vs. high-oil corn grits	1.97	> 0.05	1.65	> 0.1

Table 5 lists the amino acid contents of the germ and grits from the high-oil and the regular corn. These analyses were made on the hand-separated samples. Calculations based on the percentage composition of corn grits showed that the high-oil sample had greater amounts of all the amino acids than the regular grits sample except cystine. The regular corn germ had higher levels of all the amino acids except cystine than the high-oil corn germ. On a percentage of protein basis the high-oil grits did not contain as much lysine as the regular corn grits. On the other hand the high-oil corn germ contained more lysine than the regular corn grits.

After the whole corn samples were separated by hand, it was found that on a dry basis the regular corn contained 80.9% grits and 10.3% germ, whereas the high-oil sample yielded 76.0% grits and 14.3% germ. Table 5 lists the amino acid composition of the regular and high-oil samples supplied by the grits and germ when the amino acid values were combined in the proper proportions according to yield. These calculations do not include the amino acids supplied by the bran portions which were not analyzed. It was shown that the high-oil sample contained higher levels of all the amino acids listed, except cystine, than the regular corn sample when the calculations were based on grams of amino acid per 100 gm of corn. On a percentage of protein basis, the high-oil sample had lower amounts of histidine, methionine, lysine, phenylalanine, threonine and cystine than the regular corn.

#### DISCUSSION

The growth and protein efficiency data obtained by feeding the whole corn samples indicated that the high-oil sample was significantly better than that from the regular corn when protein levels were not adjusted. The values in table 5 do not include the amino acid content of the bran portion of the corn which was removed and discarded in the separation of the germ and grits. The bran furnished very little of the protein content of the corn; therefore, it is probable that these

since the amino acid data in table 5 indicated that the lysine content of the regular germ was lower than that of the high-oil sample (6.9 vs. 7.7%), while the threonine and tryptophan contents were approximately the same on a percentage of protein basis. Other factors, such as differences in digestibility of protein and availability of amino acids, the "balance" of all, including the non-essential amino acids, and components in the samples other than protein, may all affect the growth and protein efficiency. However, with the exception of the germ samples, good correlation was obtained between rat growth and amino acid analyses in this study.

#### SUMMARY

1. Two high-oil, high-protein corn varieties (13.0% protein with 8.6% oil and 11.5% protein with 7.0% oil) gave significant increases in growth and protein quality over that obtained from regular corn (9.3% protein with 5.1% oil) in ad libitum feeding with rats.

2. When the protein levels were adjusted to 7.5%, no significant differences in growth or protein quality were shown by the high-oil corns over the regular corn.

3. On an equal protein level of 7%, grits from one of the high-oil samples (8.6% oil) did not show significant differences in growth or protein efficiency when compared to a sample of regular corn grits. When fed at 12% protein levels, the germ from regular corn gave significantly better growth and protein efficiency than did the high-oil variety.

4. Microbiological amino acid analyses correlated rather well with rat growth tests. However, a corn germ sample containing high oil resulted in poorer growth and protein efficiency than regular corn germ which seemed to have a poorer amino acid pattern including a lower level of lysine.

#### ACKNOWLEDGMENTS

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# EFFECT OF VITAMIN B<sub>12</sub> AND AUREOMYCIN SUPPLEMENTS ON VITAMIN B<sub>12</sub> LIVER STORES AND ON THE DEVELOPMENT OF ANEMIA IN GASTRECTOMIZED RATS<sup>1,2</sup>

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Several investigators (Chow et al., '55; Watson and Florey, '55; Nieweg et al., '56) have studied the assimilation of radioactive vitamin B<sub>12</sub> by gastrectomized rats and have concluded that the vitamin is not absorbed from the intestinal tract in these animals.

In the present report, the amount of vitamin B<sub>12</sub> stored in the livers of gastrectomized rats has been measured. Since the values obtained were low when compared with those of non-operated rats fed the same diet, it became of particular interest to study the effect of oral and injected supplements of vitamin B<sub>12</sub> on the anemia which invariably develops in gastrectomized rats (Bussabarger and Jung, '36). Furthermore, since the antibiotic Aureomycin (chlortetracycline) has been found to increase the amount of free vitamin B<sub>12</sub> in the intestine of rats (Peterson et al., '53) and to increase the growth rate of several species of animals (Jukes, '55), the

<sup>1</sup>Part of the data in this paper is taken from a thesis submitted by Norma J. Long to the Graduate School of the University of California in partial fulfillment of the requirements for the Master of Science degree in Home Economics, June, 1955.

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animals were simultaneously placed on the same dietary regimen and served as controls for the experimental procedures. The animals were sacrificed after 90 days on the experimental diet. Liver tissue was removed and analyzed for vitamin B<sub>12</sub> using *L. leichmannii* ATCC 4797 (Wright et al., '48).

## RESULTS

Table 1 shows the values obtained when the liver tissue of gastrectomized rats was analyzed for vitamin B<sub>12</sub>. In two separate experiments the range and average values show that the vitamin B<sub>12</sub> content was approximately 10% of the amount present in liver tissue of non-operated control animals. This is evidence that a 90-day period on this dietary regimen results in a depletion of the vitamin B<sub>12</sub> liver content of the gastrectomized rat.

TABLE 1

*Vitamin B<sub>12</sub> in liver tissue of gastrectomized and control rats*

GROUP	NO. OF RATS	GASTRECTOMIZED RATS		CONTROL RATS	
		$\mu\text{g B}_{12}$ per gm liver		$\mu\text{g B}_{12}$ per gm liver	
		Range	Average	Range	Average
I	6	4-20	13	130-250	166
II	7	13-26	23	109-215	190

A moderate reduction in hemoglobin concentration was found in gastrectomized rats at the end of the experimental period (table 2) when values were compared to those of control animals fed the same unsupplemented diet. The addition of vitamin B<sub>12</sub> to the diet had no effect on hemoglobin values. Similar results were obtained when a hog gastric mucosal concentrate of intrinsic factor<sup>5</sup> was fed in conjunction with vitamin B<sub>12</sub> and also when vitamin B<sub>12</sub> was injected or given orally with a folic acid supplement. In experiments where Aureomycin was added to the diet, hemoglobin values were increased to the normal range. This effect of Aureomycin

<sup>5</sup> Bifactor Ser, obtained through the courtesy of Dr. K. J. Thompson of Organon, Inc., Orange, New Jersey.

livers of antibiotic-fed gastrectomized rats to be below normal values and similar to values obtained for unsupplemented gastrectomized rats (table 1). Other workers (Peterson et al., '53; Chow et al., '53) likewise found no increase in the storage of vitamin B<sub>12</sub> in antibiotic-fed animals.

#### DISCUSSION

The reduction in vitamin B<sub>12</sub> liver content occurring in gastrectomized rats can be explained on the basis that the vitamin is not absorbed from the intestinal tract in the absence of intrinsic factor (Chow et al., '55; Watson and Florey, '55; Nieweg et al., '56). The extremely low values obtained after 90 experimental days are unexpected considering the difficulties encountered in producing a vitamin B<sub>12</sub> deficiency in the normal weanling rat. The depletion of vitamin B<sub>12</sub> in gastrectomized rats apparently has no effect on blood cell production, since Aureomycin-fed animals have normal hemoglobin values associated with low vitamin B<sub>12</sub> liver stores. This would confirm the conclusion of other workers (Jukes and Williams, '54) that a vitamin B<sub>12</sub> deficiency does not greatly affect the hemopoietic process in the rat.

The results of these experiments show that the anemia occurring in gastrectomized rats on an adequate iron intake can be corrected by Aureomycin feeding. The treatment for this anemia is therefore similar to that for the anemia in the blind intestinal loop syndrome where Toon and Wangenstein ('50) found antibiotic supplements to be an effective therapy.

Although no adequate explanation is at hand, it would appear that Aureomycin could act, presumably through its effect on intestinal microflora, either to enhance the production of some unknown hemopoietic factor or to inhibit the formation of a hemolytic toxin.

#### SUMMARY

Ninety days after operation, the vitamin B<sub>12</sub> liver content of gastrectomized rats was found to be greatly reduced when



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# THE EFFECT OF FOLIC ACID ON THE USE OF GLYCINE BY THE TURKEY POULT

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Folic acid has been found to be essential for serine synthesis in microorganisms (Holland and Meinke, '49) and rat liver (Plaut et al., '50). Totter et al. ('50) found that livers of folic acid-repleted chicks were capable of converting glycine to serine more rapidly than deficient chicks. Naber et al. ('52) showed that glycine was more toxic for chicks fed a ration deficient in folic acid than for those fed a supplemented ration. Machlin et al. ('52) found folic acid and vitamin B<sub>12</sub> to be effective in counteracting glycine toxicity in chicks. More recent work has shown that a folic acid derivative, tetrahydrofolic acid, serves as a single carbon carrier in the conversion of glycine to serine in microorganisms, rats and pigeons (Blakley, '54; Elwyn et al., '55; Kisliuk and Sakami, '55; and Alexander and Greenberg, '55). The same mechanism may operate in turkey poult since the livers of poult deficient in folic acid showed a greater reduction in the ability to incorporate the alpha carbon of glycine into the beta carbon of serine than into the alpha position of serine (Vohra et al., '56).

Naber et al. ('56) were unable to simulate a toxicity of glycine in chickens by feeding compounds related to glycine, including folic acid, hydroxylic acid and serine. Dietary glycine depressed folic acid content of the tissues and folic acid deficiency decreased the glycine level in the blood.

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# THE EFFECT OF FOLIC ACID ON THE USE OF GLYCINE BY THE TURKEY POULT

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Folic acid has been found to be essential for serine synthesis in microorganisms (Holland and Meinke, '49) and rat liver (Plaut et al., '50). Totter et al. ('50) found that livers of folic acid-repleted chicks were capable of converting glycine to serine more rapidly than deficient chicks. Naber et al. ('52) showed that glycine was more toxic for chicks fed a ration deficient in folic acid than for those fed a supplemented ration. Machlin et al. ('52) found folic acid and vitamin B<sub>12</sub> to be effective in counteracting glycine toxicity in chicks. More recent work has shown that a folic acid derivative, tetrahydrofolic acid, serves as a single carbon carrier in the conversion of glycine to serine in microorganisms, rats and pigeons (Blakley, '54; Elwyn et al., '55; Kisliuk and Sakami, '55; and Alexander and Greenberg, '55). The same mechanism may operate in turkey poults since the livers of poults deficient in folic acid showed a greater reduction in the ability to incorporate the alpha carbon of glycine into the beta carbon of serine than into the alpha position of serine (Vohra et al., '56).

Naber et al. ('56) were unable to simulate a toxicity of glycine in chickens by feeding compounds related to glycine, including oxalic acid, glyoxylic acid and serine. Dietary glycine did not depress the folic acid content of the tissues and folic acid did not depress the glycine level in the blood.

Cervical paralysis has been described as a symptom of a folic acid deficiency in poults by Richardson et al. ('45), Jukes et al. ('47) and Lance and Hogan ('48). The rations used in

the expense of cornstarch. In experiments 4 and 5, 38% of acetone-extracted fishmeal replaced the soybean protein, dicalcium phosphate and calcium carbonate. In experiment 6, 30% of acid-washed casein and 0.6% of L-arginine replaced the soybean protein.

The poult s were housed in electrically heated batteries with raised wire floors and were weighed and examined at frequent intervals.

TABLE 1

*The effect of glycine, serine, betaine and folic acid upon the growth of poult s deficient in folic acid<sup>1</sup>*

SUPPLEMENT	LEVEL	PER CENT DAILY GAIN (IN %) AND SURVIVAL					
		No folic acid			Folic acid (10 mg/kg)		
		Exp. 1	Exp. 2	Av.	Exp. 1	Exp. 2	Av.
None	%	8.7 4/4 <sup>2</sup>	7.1 4/5	7.9 8/9	8.7 4/4	9.2 5/5	9.0 9/9
Glycine	4.0	1.9 2/4	4.1 4/5	3.0 6/9	9.7 4/4	8.1 5/5	8.9 9/9
DL-Serine	5.6	9.7 4/4	8.7 4/5	9.2 8/9	10.1 4/4	8.6 4/5	9.4 8/9
Betaine-HCl	0.5	7.7 4/4	8.3 4/5	8.0 8/9	9.6 4/4	8.6 5/5	9.1 9/9

<sup>1</sup> Duration—9 and 10 days in experiments 1 and 2 respectively.

<sup>2</sup> Number of survivors

Initial number

## RESULTS

In experiment 1 and 2 (table 1) glycine in the absence of folic acid caused a marked depression in growth. Three of the 9 birds in the glycine supplemented groups exhibited cervical paralysis which was very similar to that described by Richardson et al. ('45), and no cervical paralysis was noted in any of the other groups. Folic acid prevented the growth depressions as well as the cervical paralysis caused by glycine. DL-Serine at an equimolar level caused neither a growth depression nor cervical paralysis. Betaine-HCl, although used at a lower level than glycine, was not growth depressing, nor did it improve growth above the control rations. This indicates that the basal ration contained adequate methyl groups for growth, although it contained homocystine and dimethylethanolamine in an attempt to provide a low level of available methyl groups.

The glycine content of the basal ration was reduced in experiment 6 (table 4) by the use of casein and arginine as a source of amino acids. This basal ration contained 0.1 to 0.2% of glycine, by calculation. Normal poultts were used and were continued on the experiment for 34 days. Growth was reduced and mortality increased by the addition of glycine to the basal. Cervical paralysis was noted in 4 of the 6 birds in the group without glycine and folic acid. When glycine was added the poultts died sooner than in the control group and there were

TABLE 3

*Effect of glycine and folic acid on growth, survival, and incidence of cervical paralysis of poultts fed diets containing fish meal*

DURATION	GLYCINE	FOLIC ACID	DAILY GAIN	SURVIVAL <sup>1</sup>	CERVICAL PARALYSIS
days	%	mg/kg	%		% incidence
<i>Experiment 4</i>					
10	0	0	5.5	5/10	10
	2	0	..	0/10	10
	0	10	8.3	9/10	0
	2	10	7.9	7/10	0
<i>Experiment 5</i>					
9	0	0.4	7.5	1/5	0
	2	0.4	..	0/5	0
	0	10.4	7.4	5/5	0
	2	10.4	8.8	4/5	0

<sup>1</sup> Number of survivors  
Initial number

TABLE 4

*Effect of glycine and folic acid on growth, cervical paralysis, and survival of poultts fed a low-glycine diet (experiment 6)*

GLYCINE	FOLIC ACID	19-DAY DAILY GAIN	CERVICAL PARALYSIS	SURVIVAL <sup>1</sup>	
				19 days	34 days
%	mg/kg	%			
0	0	5.4	4/6	3/6	0/6
4	0	2.6	1/6	1/6	0/6
0	10	6.7	0	4/6	4/6
4	10	6.9	0	6/6	5/6

<sup>1</sup> Number of survivors  
Initial number

soybean protein, in the present studies, contained approximately 1.2% of glycine. These are rather high levels of glycine in comparison to a requirement of 0.9% (Kratzer and Williams, '48a) and might suggest that it is essential for the production of cervical paralysis. In experiment 6, however, it was shown that cervical paralysis could be produced in poult fed a ration low in glycine, and is thus actually a symptom of a folic acid deficiency. This fact gives further support to the theory that glycine is toxic by creating a folic acid deficiency.

The cervical paralysis seems to be a symptom of a chronic deficiency since poult which were depleted when hatched and fed an extremely deficient ration showed less cervical paralysis than poult fed higher levels of folic acid or less glycine. This is analogous to a deficiency of pantothenic acid in which early mortality may prevent the development of typical symptoms (Kratzer and Williams, '48b).

#### SUMMARY

In poult fed rations low in folic acid, glycine caused depressed growth, increased mortality and cervical paralysis. The effects could be prevented by supplementing the rations with folic acid. DL-Serine at an equimolar level had no adverse effect upon the birds. Poult fed a ration deficient in glycine as well as folic acid also developed cervical paralysis. This indicates that the cervical paralysis is a result of a deficiency of folic acid rather than an excess of glycine.

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# THE ADDITION OF NON-IONIC SURFACE-ACTIVE AGENTS OF THE POLYOXYETHYLENE TYPE TO THE DIET OF THE HAMSTER, THE MOUSE AND THE DOG<sup>1</sup>

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The non-ionic surface-active agents, such as the sorbitan and polyoxyethylene derivatives of fatty acids, have a number of attractive uses in food technology, particularly as emulsifiers. It is important, therefore, to establish the effects of these compounds, in amounts proposed for technological purposes, on the nutrition and health of the consumer. Data presented in the literature can be interpreted to mean that these compounds should not be classified as substitutes for fats but rather be considered as food additives, compounds which could be used in relatively small amounts whenever or wherever they develop technological advantage.

One of the most difficult tasks presented to food technologists is the determination of safety, particularly of compounds that are not recognized as normal to foods and work on this problem has been done by Bourke and Fitzhugh, '53; Chow et al., '53; Culver et al., '51; Harris et al., '50; Jones et al., '48; Krantz et al., '51; Schweigert et al., '51; Wang et al., '50; Allison et al., '52. The task is difficult, in part, because any substance, whether it be classified as inert, as a food,

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all of its parts increase in weight or volume in a definite pattern. This pattern of growth was determined by measuring not only the weight of the whole animal but also some of its parts. The process of growth is a function of food intake and its utilization, a function which was expressed as food efficiency. Abnormalities were detected by histopathological techniques.

TABLE 1  
*Basal diets*

INGREDIENT		INGREDIENT	
	gm		mg/1000 gm of dry diet
Diet A			
Casein (vitamin-free)	180	Thiamine	10
Sucrose	134	Riboflavin	20
Dextrose	202	Pyridoxine	10
Dextrin	140	Vitamin K	15
Lard	241	Niacin	80
Salt mixture <sup>1</sup>	40	Pantothenic acid	80
Agar	33	Inositol	200
Cod liver oil	20	Folic acid	0.5
Liver powder	10	Biotin	0.5
	1000	Alpha-tocopherol	40 units
		PABA	80
Water	1400	Choline	2000
	2400	Ascorbic acid	2
		Vitamin A	40,000 units
		Vitamin D	4,000 units
Diet B			
Casein (vitamin-free)	250	Thiamine	2.0
Sucrose		Riboflavin	1.6
Dextrose	366	Nicotinic acid	16.0
Dextrin	187	Calcium pantothenate	13.0
Lard	153	Pyridoxine	1.0
Salt mixture <sup>1</sup>	17	Choline	1000.0
Agar	27	2-Methylerythritolquinone	0.0050
	1000	Alpha-tocopherol	2.00
		Biotin	0.0
Water	1400	Folic acid	0.0
	2400	Vitamin A	7700 units
		Vitamin D	1100 units

<sup>1</sup> Wesson (1952).



*Diet and the disease "Wet Spot"*. It is possible that many experiments where synthetic diets are fed to hamsters may be complicated by the disease "Wet Spot" which is characterized primarily by a wet posterior end, with bloody diarrhea and inanition. A private communication from Ben-Menachem at Hebrew University, Jerusalem, suggests that the disease may be similar to the obscure and fatal "Wet Tail" referred to by Hindle (see Worden, '47). Hamsters fed diet A grew and were in excellent condition provided they were free from this disease. Exposure to "Wet Spot" was impossible to avoid, however, and occurred when animals were placed in cages that had contained sick animals or that were adjacent to sick animals.

Since "Wet Spot" was seldom found in animals fed the commercial "Fox Chow," variations were made in diet A to include this and certain natural foodstuffs. Addition of 30% of "Fox Chow," yeast, or alfalfa meal to diet A reduced the incidence of "Wet Spot," and reduced mortality from close to 100% to under 50%. Decreasing the fat in diet A by one-half, to approximate more nearly the low fat content (3%) of "Fox Chow," did not protect the animals from the disease entirely, but delayed its onset. Much work needs to be done to characterize the disease and the protective effect of foods, but these preliminary observations are mentioned at this time since the disease may be prevalent in other colonies of hamsters. Our work was continued using "Fox Chow" as the basal diet.

*Surface-active agents in the "Fox Chow" diet*. Two experiments were carried out with the same general experimental pattern. Weanling hamsters were divided at random into groups of 12 animals each, the groups having the same average body weight, and fed "Fox Chow" alone or "Fox Chow" containing varying amounts, in terms of percentage weight, of the surface-active agents Myrj 45, Myrj 52, Tween 60, or 1% of vegetable fat. In one experiment, hamsters weighing approximately 27 gm were divided into 4 groups fed "Fox Chow" containing 0, 2.5, 5 or 10% of Myrj 52, respectively.

TABLE 2

*Food efficiency and body weights in hamsters fed "Fox Chow" or "Fox Chow" containing Myrj 45, Myrj 52, Tween 60 or a vegetable fat*

DIET	FOOD EFFICIENCY <sup>1</sup> FIRST 6 WEEKS	BODY WEIGHT <sup>2</sup> MONTHS ON DIET			
		0	3	6	12
		gm	gm	gm	gm
<i>Experiment 17</i>					
Fox Chow	...	27	76	87	101
Myrj 52, 2.5%	...	26	79	98	108
Myrj 52, 5%	...	27	79	95	100
Myrj 52, 10%	...	27	81	94	101
<i>Experiment 19</i>					
Fox Chow	0.20	50	98	110	106
Vegetable fat, 1%	0.25	43	90	103	94
Myrj 45, 1%	0.26	52	96	110	99
Myrj 45, 5%	0.19	52	90	103	102
Myrj 52, 1%	0.22	52	93	103	96
Myrj 52, 5%	0.21	53	92	109	102
Tween 60, 1%	0.24	53	97	111	108
Tween 60, 5%	0.19	53	98	110	103
<i>Experiment 20</i>					
Fox Chow	0.19	40	100	103	101
Vegetable fat, 1%	0.19	40	96	100	104
Myrj 45, 1%	0.20	40	107	104	105
Myrj 45, 5%	0.20	40	100	104	108
Myrj 52, 1%	0.20	40	98	104	101
Myrj 52, 5%	0.18	40	94	99	96
Tween 60, 1%	0.19	40	97	99	94
Tween 60, 5%	0.19	40	98	101	104
<i>Experiment 21</i>					
Fox Chow A	0.16	49	100	107	104
Fox Chow B	0.16	49	94	108	104
Myrj 45, 1%	0.16	49	98	104	101
Myrj 45, 5%	0.16	49	98	99	99
Myrj 52, 1%	0.17	49	97	113	100
Myrj 52, 5%	0.14	49	87	97	90
Tween 60, 1%	0.16	49	95	110	107
Tween 60, 5%	0.16	49	97	100	104

<sup>1</sup> Grams weight gained per gram of food consumed.

<sup>2</sup> Twelve animals in each dietary group.

TABLE 3

*Average body weight and organ weights of hamsters fed control and experimental diets for 12 to 13 months*

DIET	NO. ANIMALS	BODY WEIGHT	HEART	LIVER	KIDNEY	SPLEEN
		gm	gm	gm	gm	gm
<i>Experiment 17 (Started 3/19/52)</i>						
Fox Chow (Control)	9	101	0.403	3.93	0.767	0.078
Myrj 52, 2.5%	11	108	0.430	3.99	0.815	0.089
Myrj 52, 5%	8	100	0.476	3.68	0.859	0.110
Myrj 52, 10%	11	101	0.400	3.91	0.851	0.081
<i>Experiment 19 (Started 2/16/53)</i>						
Fox Chow (Control)	11	106	0.398	4.28	0.899	0.076
Vegetable fat, 1%	12	94 <sup>1</sup>	0.357 <sup>2</sup>	3.80	0.752 <sup>1</sup>	0.067
Myrj 45, 1%	10	99	0.377	4.05	0.869	0.079
Myrj 45, 5%	12	102	0.380	4.24	0.893	0.070
Myrj 52, 1%	11	96	0.401	3.84 <sup>1</sup>	0.911	0.071
Myrj 52, 5%	11	103	0.387	4.04	0.886	0.072
Tween 60, 1%	12	108	0.414	3.99	0.967	0.076
Tween 60, 5%	12	103	0.396	3.94	0.955	0.072
<i>Experiment 20 (Started 3/23/53)</i>						
Fox Chow (Control)	12	101	0.413	3.42	0.815	0.082
Vegetable fat, 1%	10	104	0.411	3.45	0.724	0.076
Myrj 45, 1%	10	105	0.460	3.73	0.910	0.079
Myrj 45, 5%	10	108	0.403	4.23 <sup>1,2</sup>	0.841	0.072
Myrj 52, 1%	11	101	0.398	3.58	0.796	0.075
Myrj 52, 5%	11	97	0.376	3.49	0.776	0.073
Tween 60, 1%	12	94	0.379 <sup>1</sup>	3.37	0.776	0.074
Tween 60, 5%	9	104	0.397	3.61	0.970	0.088
<i>Experiment 21 (Started 6/11/53)</i>						
Fox Chow (Control)	11	104	0.420	3.91	0.824	0.100
Fox Chow (Control)	12	105	0.418	3.97	0.831	0.101
Myrj 45, 1%	11	101	0.404	3.63	0.780	0.085
Myrj 45, 5%	11	99 <sup>1</sup>	0.375 <sup>1</sup>	3.59	0.733 <sup>1</sup>	0.086
Myrj 52, 1%	12	103	0.410	3.74	0.874	0.092
Myrj 52, 5%	10	99 <sup>1</sup>	0.383	3.65	0.786	0.086
Tween 60, 1%	12	107	0.452	3.66	0.822	0.102
Tween 60, 5%	12	104	0.413	3.92	0.819	0.095

<sup>1</sup> Significant at  $P < 0.05$  confidence level.

<sup>2</sup> Only weight significantly different from control (at  $P < 0.05$  confidence level) when values expressed per 100 gm body weight.

The third type of pathology was concerned with the kidneys. This pathology could be divided into a primary type characterized by the formation of hyaline casts in the renal tubules and a secondary type characterized by hyaline tubule casts accompanied by chronic interstitial nephritis. The data indicate that whenever diarrhea was present the incidence of casts and chronic interstitial nephritis generally increased

TABLE 4

*Pathologies in hamsters fed "Fox Chow" or "Fox Chow" containing vegetable fat, Myrj 45, Myrj 52, or Tween 60*

DIET	NUMBER ANIMALS	DEAD AT 1 YEAR	DIARRHEA	MILD TESTICULAR ATROPHY	CASTS <sup>1</sup>	CIN <sup>1</sup>
Fox Chow	36	2	—	1	9	2
Vegetable fat, 1%	24	2	—	1	8	1
Myrj 45, 1%	36	5	—	1	4	1
Myrj 45, 5%	36	3	—	0	12	0
Myrj 52, 1%	36	3	—	1	10	3
Myrj 52, 5%	36	3	+	0	17	5
Tween 60, 1%	36	0	—	0	11	0
Tween 60, 5%	36	3	+	1	18	6
Fox Chow	12	3	—	1	2	0
Myrj 52, 2.5%	12	1	—	1	4	1
Myrj 52, 5%	12	3	+	4	8	1
Myrj 52, 10%	12	1	+	7	10	6

<sup>1</sup> Casts and chronic interstitial nephritis (CIN) in kidney.

significantly above the controls and other groups fed surface-active agents without resulting diarrhea (see table 4). Diarrhea was obvious in animals fed 5 and 10% of Myrj 52 and 5% of Tween 60 and absent in animals fed 1 or 2.5% of Myrj 52, 1% of Tween 60 and 1 or 5% of Myrj 45.

In order to investigate further the cause of increased kidney pathology in hamsters fed higher concentrations of Myrj 52 and Tween 60, 4 groups of 10 young male hamsters each were fed, ad libitum, diets of "Fox Chow" alone or "Fox Chow" containing 5% of Myrj 45, 5% of Myrj 52, or 5% of Tween 60 for one week. For the next three days, while the animals were fed the same diets, food and water intakes were measured

organs weighed and tissues sectioned, stained and examined for histopathology as in the case of the hamsters.

*Environmental factors.* High mortality was observed in early experiments in groups of mice fed diet A containing 15% of Myrj 52 or Tween 60. This high mortality was concurrent with an erythema and loss of hair. Data were obtained to suggest that these conditions were due to direct skin contact with the experimental diets and resulting cannibalism. Although similar effects were produced by the control diet alone when it was rubbed onto the skin of the animals, the

TABLE 5  
*Average body weight and organ weights of mice fed various amounts of Myrj 45, Myrj 52 or Tween 60 in diet A for 3 to 4 months<sup>1</sup>*

DIET	NO. ANIMALS	BODY WEIGHT	HEART	LIVER	KIDNEYS	SPLEEN
		gm	gm	gm	gm	gm
Diet A (Control)	13	38.7	0.156	1.61	0.526	0.213
+ Myrj 45, 5%	9	41.6	0.182	1.70	0.645	0.235
+ Myrj 45, 10%	12	42.3	0.176	1.83	0.605	0.225
+ Myrj 52, 2.5%	11	43.4	0.160	1.74	0.537	0.236
+ Myrj 52, 5%	10	40.3	0.163	1.68	0.554	0.251
+ Myrj 52, 10%	10	40.3	0.168	1.64	0.538	0.184
+ Tween 60, 2.5%	6	42.1	0.186	1.82	0.576	0.196
+ Tween 60, 5%	9	41.2	0.173	1.68	0.552	0.202
+ Tween 60, 10%	9	42.0	0.161	1.92	0.550	0.234

<sup>1</sup> There is no difference, at the  $P \leq 0.05$  confidence level, between the average body weights and organ weights of any of the experimental groups and the control.

condition was aggravated when the food contained the surface-active agents. Lowering of mortality and improvement of the skin condition occurred when wood shavings were kept in the wire or metal-bottom cages in which the mice were housed and when food was available only from overhead wire baskets. Therefore all subsequent experiments were conducted in this fashion.

*Body weight gain and organ weights.* There were no outstanding differences in average body weights of animals fed the various experimental diets over periods of from three to 4 months, nor did the organ weight of these animals

mononuclear perivascular cuffing in the bladder, which approximated early cystitis. Eight of the 9 mice fed 5% and 9 of the 12 fed 10% of Myrj 45 showed signs of the generalized colony infection. Two mice fed 5% of Myrj 45 and none in the 10% Myrj 45 group had marked infiltration of the liver, with intranuclear inclusion bodies. No pathological conditions attributable to the presence of Myrj 45 in the diet were evident. Five of the 11 mice fed 2.5%, 6 of the 10 fed 5%, and 8 of the 10 fed 10% of Myrj 52 demonstrated pathology that was associated in whole or in part with the colony infection. There were no abnormalities attributable to the feeding of Myrj 52. In the experiments involving Tween 60, 5 of the 6 mice fed 2.5% of this surface-active agent, 7 of the 9 fed 5% and 7 of the 9 fed 10% had evidence of the colony infection. Two animals, one fed 2.5% and one fed 10% of Tween 60, evidenced a mild gastritis. There were no pathological conditions specific for Tween 60.

#### EXPERIMENTS WITH BEAGLES

Weanling beagle puppies were fed an agar gel diet B (table 1) containing 0, 5, or 10% (dry basis) of Myrj 45, Myrj 52 or Tween 60. The basal diet, containing 25% of casein, has been used for a number of years to grow and maintain dogs. Protein efficiencies are recorded in table 7. More data are needed to determine the variation in protein efficiencies of beagle puppies but these values are of the same order of magnitude as those determined previously on this diet in our laboratories, and the variation is small when it is considered that each value represents a single animal. None of the animals developed diarrhea on any of these diets. One animal fed 5% of Myrj 45 for approximately one year and two receiving 10% of Myrj 45 for 40 weeks were autopsied and the same group of tissues listed for hamsters and mice were examined for histopathology. No significant pathology attributable to the feeding of Myrj 45 was found. Two dogs, one male and one female, receiving 5% of Myrj 52 were autopsied at the end

because of the lack of protection by the diet against a disease we have called "Wet Spot." Partial protection could be obtained by adding alfalfa meal, certain yeasts or "Fox Chow" to the synthetic diet. For that reason, "Fox Chow" was used as the basal diet. Even with some asymmetry of stresses between groups may be one explanation of the fact that in triplicate experiments some significant differences ( $P \leq 0.05$ ) occurred in one member of the triplicate which disappeared when the data were considered as a whole.

The addition of Myrj 45, Myrj 52 or Tween 60 at varying concentrations to the diet of hamsters, mice or dogs did not alter growth or food efficiency. Myrj 45 did not produce diarrhea in any of the animals or any abnormalities that could be attributed to this surface-active agent *per se*. The possibility that 5% of Myrj 45 could be considered the upper limit for feeding surface-active agents to hamsters might be argued because in a single experiment there was a significant fall in body weight in year-old animals below the "Fox Chow" control, a significance, however, that disappeared when triplicate experiments were analyzed. A reduction in growth and food efficiencies was reported by Schweigert et al. ('50) for hamsters fed 5 and 15% of Myrj 45 in place of an equivalent amount of lard in a semi-synthetic diet. The gastrointestinal disturbances found by these authors in hamsters fed Myrj 45 and the accompanying pathologies (see Wang et al., '50) were not observed in any of our animals. Possibly there were added stresses in their experiments, such as a nutritional imbalance due to substitution of Myrj 45 for lard. Recently Krehl, Cowgill and Whedon ('55) concluded from their studies on the effects of polyoxyethylene esters in the diet of the rat and the cat that Myrj 45 up to 20% of the diet, or the oxyethylene moiety at a level of 6%, had no deleterious effect.

Diets containing 5% of Myrj 52 or Tween 60 did produce diarrhea in hamsters. Higher concentrations of these surface-active agents up to 10 and 20% were necessary to produce diarrhea in mice or dogs. Increase in the incidence of kidney casts and of chronic interstitial nephritis occurred in hamsters

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